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ISOLATION OF THE NICOTINAMIDE FORMED FROM ASPARAGINE AND GLUTAMIC ACID

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(Received for publication, December 21, 1943)

The formation in heated mixtures of asparagine and glutamic acid of a substance that shows certain biological and chemical properties of nicotinamide was reported in earlier papers (1-3). This substance has now been isolated and identified as nicotinamide itself.

EXPERIMENTAL

The optimal conditions previously described were followed in carrying out the reaction between asparagine and glutamic acid on a relatively large scale, a solution containing 50 gm per liter of each of the two amino acids and 0.15 gm per liter of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ was heated in a water bath with aeration for 9 days. The solution was then concentrated *in vacuo* to about one-quarter of its original volume and extracted with ether for 48 hours. The ether extract was dried, taken up in a small volume of 0.05 M phosphate, pH 7, filtered, and again extracted with ether for 48 hours. After removal of the solvent from the second ether extract, a heavy brown oil remained.

The various fractions obtained in this procedure were assayed for nicotinamide activity by the method previously described (3) that was based on the growth of *Bacterium dysenteriae*. In a typical run, starting with 1 liter of asparagine-glutamate solution, 66.2 mg of nicotinamide were formed. After the first ether extraction 0.8 mg (1.2 per cent) remained in the water solution and after the second, 3.6 mg (5.4 per cent), the second ether extract contained 57.2 mg (86 per cent). The apparent loss of 7.4 per cent is within the limits of error of the assays. The total dry weight of the second ether extract was 125 mg.

Because of the large amount of amino acids required for this preparative work, commercial amino acids were used. Previous work had shown that nicotinamide activity is formed not only from the highly purified natural amino acids, but also from the synthetic compounds (1). Assay of an ether extract of an unheated control solution of the commercial asparagine and glutamic acid showed that less than 0.01 mg per liter of nicotinamide was present.

For further purification, the combined crude concentrates from several

runs, containing in all 345 mg of nicotinamide by assay, were dissolved in dilute phosphate, pH 7, and extracted with ether for 24 hours. The extracted material was dried and then refluxed with about 80 ml of boiling benzene for 20 minutes. After the solution was decanted, filtered, and cooled, a mixture of yellow oil and crystals separated. This material was extracted again with hot benzene, about 20 ml being used for 60 mg. The benzene solution was concentrated to about 8 ml and allowed to cool. The resulting precipitate was twice recrystallized by the same procedure and finally yielded 36 mg of white crystals that melted at 125–126°. These were sublimed *in vacuo* at 95° and the sublimate was recrystallized once more from benzene. Yield 27.5 mg, m p 126–127°, m p, mixed with commercial nicotinamide, 126–127°.

Analysis—Found, N 23.26, theory, N 22.95

Reworking of the insoluble residues from the benzene extraction of the mother liquors by reextraction from water, followed by repeated recrystallizations from benzene, or by alternate recrystallization and sublimation, yielded a further 176 mg of crystals with a melting point of 125–126°. A total of 212 mg of nicotinamide, or 61 per cent of the estimated nicotinamide content of the crude starting material, was isolated in pure form.

The hydrochloride was prepared by the addition of an excess of alcoholic hydrochloric acid to a 10 per cent alcoholic solution of this material and as recrystallized once from alcohol. M p 226.5–227°, m p, mixed with hydrochloride prepared from commercial nicotinamide, 226–227°.

Analysis—Found C 45.6, H 4.25, N 17.47, Cl 22.16

Theory " 45.45, " 4.45, " 17.66, " 22.36

The chloroaurate was prepared as described by Elvehjem *et al* (4). M p 204–205°, m p, mixed with chloroaurate prepared from commercial nicotinamide, 204–205°.

DISCUSSION

These experiments demonstrate conclusively that nicotinamide is formed in the reaction between asparagine and glutamic acid. Since over half of the amount expected from biological assays was isolated by a method that involved repeated fractional recrystallizations, and since chemical and biological analyses of the crude ether-extractable material were in complete agreement, the evidence appears definite that the nicotinamide produced is responsible for all of the observed biological activity.

The active products formed when certain other amino acids (3) are heated with asparagine have not been isolated. However, chemical analysis of the ether-soluble material from the reaction between methionine and aspara-

gine indicated that in this instance also nicotinamide was produced (2). In view of the certainty that nicotinamide is the product in one case, and the probability that it is in another, and considering the well known biological specificity of this growth factor (5-7), there seems little doubt that in all of the reactions described in an earlier publication (3) between asparagine and other amino acids the biologically active substance formed is the same.

SUMMARY

The nicotinamide-like substance that is produced when asparagine and glutamic acid are heated together has been isolated and identified as nicotinamide.

The author is indebted to Mr William Saschek of Columbia University for the microanalyses herein reported.

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STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

XIII ANTICOAGULANT ACTIVITY AND STRUCTURE IN THE 4-HYDROXYCOUMARIN GROUP*

By RALPH S. OVERMAN, MARK ARNOLD STAHMANN, CHARLES FERDINAND HUEBNER, WILLIAM R. SULLIVAN, LEONARD SPERO,
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(Received for publication, December 6, 1943)

Shortly after it was established that 3,3'-methylenebis(4-hydroxycoumarin) is the causative agent of the hemorrhagic sweet clover disease of cattle (April, 1940), it was found that the hypoprothrombinemia that characterizes this disease could also be induced by analogous compounds (1-3)

In a preliminary announcement made in February, 1942 ((3) p. 953), we outlined the scope of experiments under way designed to show the relationship of structure in the 4-hydroxycoumarin group to hypoprothrombinemia-inducing (anticoagulant)¹ capacity. In the interim work by Jansen and Jensen (4), Lehmann (5), and Fantl (6) has appeared which reports on the activity of twelve of the compounds included in this study. Although the methods of assay and of expressing the results used by them differ from ours, the results are qualitatively in agreement.

Practically all of the bis 4-hydroxycoumarins and their derivatives, as well as most of the 4-hydroxycoumarin compounds used in this study were synthesized by methods described in a recent series of papers (7-12).

Assay Method

The methods used have been described in previous papers of this series ((13), (3) specifically pp. 943-944). All the assays were made with standardized, susceptible rabbits, 2 to 3 years old, weighing about 2.5 kilos. Only those rabbits were used that showed an increase in the pro-

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station and supported since July, 1940, through special grants from the Graduate Research Committee of the University, Office of Dean E. B. Fred, and the Wisconsin Alumni Research Foundation.

¹ The term anticoagulant is used in the general sense that 3,3'-methylenebis(4-hydroxycoumarin) is an agent which, after action *in vivo*, impairs or prevents the coagulation of blood. It and the other compounds included in this study do not affect the clotting power when added *in vitro* to blood or plasma.

thrombin time of the 12.5 per cent plasma (1 part of plasma, 7 parts of saline solution) greater than 20 seconds 72 hours after feeding 2.5 mg of 3,3'-methylenebis(4-hydroxycoumarin). The assay rabbits were re-standardized periodically.

Each compound was fed in gelatin capsules to a minimum of three rabbits at three or more of the following levels: 2.5, 5.0, 50, 200, and 500 mg, and blood samples were taken for the prothrombin determination 24, 48, and 72 hours after administration. Unless otherwise indicated the time of maximum increase was 72 hours after feeding the compounds.

The average increase in the prothrombin time of the 12.5 per cent plasma over normal was tabulated for each level and from the data an abbreviated and more useful expression, the relative anticoagulant index, was calculated. This index serves as an approximate basis for the comparison of activity.

The relative anticoagulant index is defined as the seconds increase in prothrombin time $\times 0.0186$ per mm of test compound. The factor 0.0186 is included to make the index of 3,3'-methylenebis(4-hydroxycoumarin), the reference standard, equal to 100 at the dose of 0.75 mg. Thus 1.5 mg of 3,3'-methylenebis(4-hydroxycoumarin) prolong the prothrombin time of the 12.5 per cent plasma 19 seconds (Table I) and the index is $(19 \times 0.0186 \times 336) / 1.5 = 79$. 336 is the molecular weight of 3,3'-methylenebis(4-hydroxycoumarin).

This index is a measure of the efficiency of the anticoagulant. Since the efficiency of action is decreased at low levels by a threshold effect and at high levels by incomplete absorption, a certain dosage level will give the maximum index ((3) Fig 1, p. 944). The variance of the relative anticoagulant index of 3,3'-methylenebis(4-hydroxycoumarin) for seven different dosage levels is shown in Table I. Each dose was fed to eight or more rabbits a minimum of six times.

It is evident that the most efficient dose is 0.75 mg, the index being 100. The standard error for the increase in prothrombin time over normal at each of the dosage levels (Table I) gives an indication of the variation between these and all subsequent assays. For all the other compounds tested only the maximum index and the level at which it occurred are given in Tables II to VIII. Since the detectable dose of 3,3'-methylenebis(4-hydroxycoumarin) is about 0.40 mg in the rabbits used in this work, doses higher than 500 mg were usually not tested, because it is arbitrarily considered that, to be biologically significant, activity of a single oral dose should be detectable at that level.

Anticoagulant Activity of Various Classes of Compounds

Bis-4-hydroxycoumarins—The condensation of 2 moles of 4-hydroxycoumarin with aldehydes (8) has provided a series of bis-4-hydroxycoumarins that differ from the parent substance 3,3'-methylenebis(4-

hydroxycoumarin) in that the methylene group has 1 hydrogen atom substituted by an alkyl or aryl group. These compounds are represented

TABLE I
Anticoagulant Activity of 3,3'-Methylenebis(4-hydroxycoumarin) at Different Dosage Levels

3,3'-Methylenebis(4-hydroxycoumarin)	Increase in prothrombin time over normal, and standard error	Relative anticoagulant index and standard error
mg	sec	
0.37	35 ± 1.5*	59 ± 25
0.75	12 ± 3.4†	100 ± 28
1.50	19 ± 4.1†	79 ± 17
3.00	37 ± 2.9‡	78 ± 6
6.00	57 ± 8.8‡	59 ± 9
50.0	65 ± 9.0‡	8 ± 1
200.0	83 ± 2.8‡	3 ± 0.1

* Maximum increase 24 hours after feeding

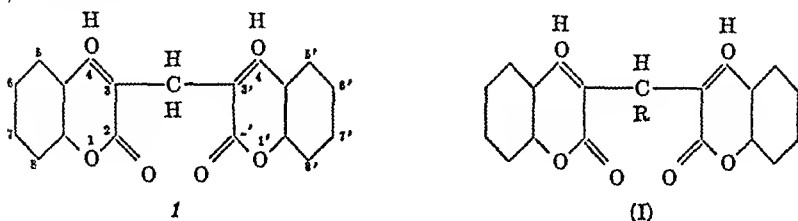
† 48 hours after feeding

‡ 72 hours after feeding

TABLE II
Anticoagulant Activity of Bis-4-hydroxycoumarins

Compound	Relative anticoagulant index	Dosage for maximum index
		mg
1 3,3'-Methylenebis(4-hydroxycoumarin)	100	0.75
2 3,3'-Ethylidenebis(4-hydroxycoumarin)	24	5.0
3 3,3'-Propylidenebis(4-hydroxycoumarin)	32	5.0
4 3,3'-Butylidenebis(4-hydroxycoumarin)	8.6	5.0
5 3,3'-Isobutylidenebis(4-hydroxycoumarin)	2.4	50
6 3,3'-Pentylidenebis(4-hydroxycoumarin)	3.5	200
7 3,3'-Isopentylidenebis(4-hydroxycoumarin)	1.5	50
8 3,3'-Hexylidenebis(4-hydroxycoumarin)	3.0	200
9 3,3'-Benzylidenebis(4-hydroxycoumarin)	0.4	500
10 3,3'-(p-Methoxybenzylidene)bis(4-hydroxycoumarin)	0.2	500
11 3,3'-(p-Hydroxy-m methoxybenzylidene)bis(4-hydroxycoumarin)	0.4	500
12 3,3'-(m,p-Methylenedioxybenzylidene)bis(4-hydroxycoumarin)	0.9	500
13 3,3'-(p-Dimethylaminobenzylidene)bis(4-hydroxycoumarin)	0.3	500
14 3,3'-Phenylethylidenebis(4-hydroxycoumarin)	1.1	200
15 3,3'-Phenylpropylidenebis(4-hydroxycoumarin)	1.3	200
16 3,3'-Carboxymethylidenebis(4-hydroxycoumarin)	0.2	200
17 3,3',3'',3'''-Adipylidenetetrakis(4-hydroxycoumarin)	0.1	500
18 3,3'-Thiobis(4-hydroxycoumarin)	5.3	5.0

by the general formula (I) The anticoagulant activity of this series is given in Table II

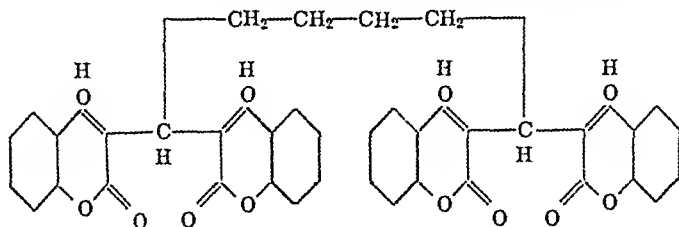


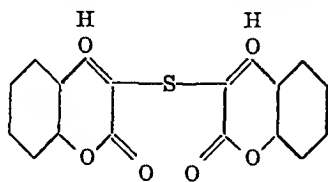
All the comparisons of activity are made with 3,3'-methylenebis(4-hydroxycoumarin) which henceforth will usually be designated as *1*. Likewise the numbers in italic type will be used in the text to refer to the compounds listed in Tables II to VIII. For example, the maximum index for *1* is 100 and for *8* is 3. Then *8* is 3/100 or approximately 1/30 as active as *1*. It is to be emphasized that the maximum index of each compound is taken for the comparison. The formula of *1* and all other 4-hydroxycoumarins is given only in the enol form.

As the hydrogen atom on the methylene group is substituted by a 1st, or a 2 carbon atom residue, *2* and *3*, the activity decreases to $\frac{1}{2}$ that of *1*, a 3 carbon chain, *4*, decreases it to 1/10 and a 4 or 5 carbon chain, *6* and *8*, to 1/30 that of *1*.

Branching in the carbon chain, *5* and *7*, decreases the activity to about $\frac{1}{2}$ that of the corresponding straight chain compound. The introduction of a phenyl group, *9*, on the methylene carbon bridge reduces the activity to 1/300 that of *1*. Substitution into this aromatic group of hydroxyl or amino residues, *10*, *11*, *12*, and *13*, affects the activity of the parent substance, *9*, only slightly. When the phenyl group is located farther from the bridge methylene carbon as in *14* and *15*, the activity becomes greater than in the parent substance *9*. Substitution of a carboxyl group on the methylene bridge, *16*, greatly increases the solubility but the activity decreases to 1/500 that of *1*.

When 2 molecules of *1* are joined through their methylene groups by a 4 carbon atom bridge, *17*, the activity decreases to 1/1000 that of *1*. In

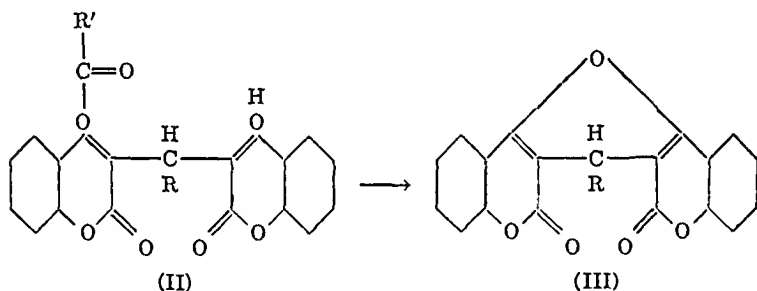




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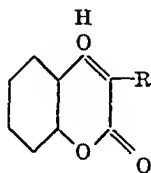
3,3'-thiobis(4-hydroxycoumarin), 18, the methylene group of 1 has been replaced by a sulfur atom. This compound exhibits 1/20 the activity of 1.

When monoesters, organic or inorganic (II), and monoglucosides of the bis-4-hydroxycoumarins are neutralized, immediate formation of the biologically inactive anhydride compounds (III) occurs (9, 12). Since conjugations with sulfuric acid or *d*-glucuronic acid are routes for the detoxication of enols *in vivo*, it is conceivable that the bis-4-hydroxycoumarins might be inactivated via this mechanism. Anhydride formation



is facilitated by the substitution of the methylene carbon atom (9). Dehydration proceeds most readily in compound 9, which is also the most inactive compound of its class. Hence, efficient, rapid detoxication via anhydride formation may account for the decrease in anticoagulant activity of the bis-4-hydroxycoumarins as the methylene carbon atom is substituted.

3-Substituted 4-Hydroxycoumarins—3,3'-Methylenebis(4-hydroxycoumarin) is a 3-substituted 4-hydroxycoumarin in which the substituent group contains another 4-hydroxycoumarin residue. In view of this, a number of 3-substituted 4-hydroxycoumarins were prepared (7). The anticoagu-



(IV)

lant activity of these compounds is given in Table III. They have the general structure of (IV). The highest activity among the simple 3-alkyl- and 3-aryl-4-hydroxycoumarins is exhibited by 3-hexadecyl-4-hydroxy-

TABLE III
Anticoagulant Activity of 3 Substituted 4-Hydroxycoumarins

Compound	Relative anticoagulant index	Dosage for maximum index
		mg
19 4-Hydroxycoumarin	0.12	200
20 3-Methyl-4 hydroxycoumarin	0.10	500
21 3-Ethyl-4 hydroxycoumarin	0.05	1000
22 3-Propyl-4-hydroxycoumarin	0.08	500
23 3-Isopropyl-4 hydroxycoumarin	0.03	500
24 3-Butyl-4 hydroxycoumarin	0.09	200
25 3-Amyl-4-hydroxycoumarin	0.24	200
26 3-Hexadecyl-4 hydroxycoumarin	1.5	50
27 3-Phenyl 4 hydroxycoumarin	1.2	50
28 3-Benzyl 4 hydroxycoumarin	1.1	50
29 3-Acetyl-4 hydroxycoumarin	1.0	50
30 3-Benzoyl-4 hydroxycoumarin	Inactive at	500
31 3-Carboethoxy-4 hydroxycoumarin	0.12	200
32 3-Cyano 4 hydroxycoumarin	Inactive at	500
33 3-[6-Oxo(1)benzopyran(4,3-b)(1)benzopyran-7-yl]-4-hydroxycoumarin	6.5	50
34 3-[6-Oxo-10-hydroxy(1)benzopyran(4,3-b)(1)benzopyran-7-yl]-4 hydroxycoumarin	Inactive at	500
35 6-Oxo-7-benzoyl-1-methyl(1)benzopyran(4,3-b)(1)benzopyran	" "	500
36 6-Oxo-7-salicylmethyl(1)benzopyran(4,3-b)(1)benzopyran	0.11	500
37 3-Oximino-4 hydroxycoumarin	Inactive at	500
38 3-Nitro-4 hydroxycoumarin	" "	500
39 3-Bromo 4 hydroxycoumarin	" "	500
40 3-(α -Methyl β -acetylmethyl) 4 hydroxycoumarin	0.8	50
41 3-(α -Phenyl β -benzylethyl)-4 hydroxycoumarin	6.0	5
42 3-(α -Phenyl β -acetylmethyl) 4 hydroxycoumarin	21	5
43 3-[α -(p-Methoxyphenyl) β -acetylmethyl]-4 hydroxycoumarin	50	5
44 3-[α -(p-Hydroxy m-methoxyphenyl) β -acetylmethyl]-4 hydroxycoumarin	12	5

coumarin, 26, 3-phenyl-4-hydroxycoumarin, 27, and 3-benzyl-4-hydroxycoumarin, 28. They are about 1/80 as potent as 1. As the size of the 3-substituent decreases, the activity is reduced. 4-Hydroxycoumarin, 19, has 1/800 the activity of 1. Minimum activity in this class is reached with

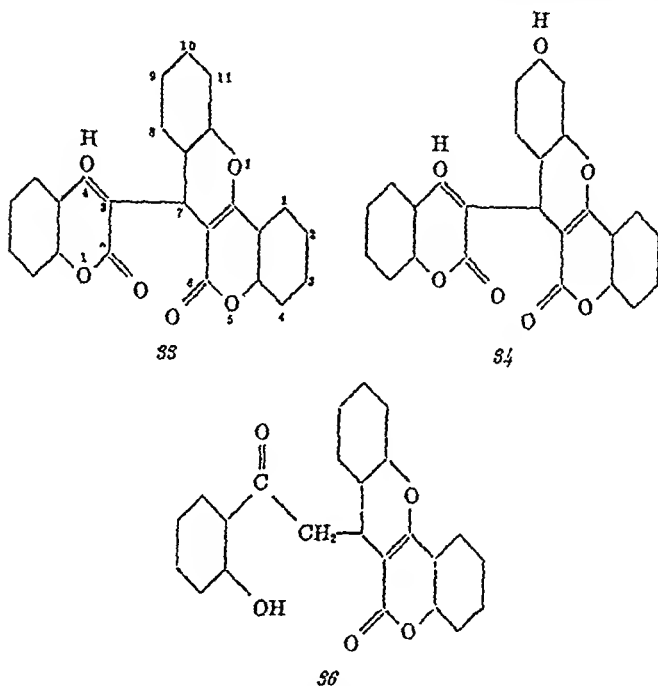
3-ethyl-4-hydroxycoumarin, 21, and 3-propyl-4-hydroxycoumarin, 22
It should be indicated that the maximum activity of compounds 19 to 29
in this class is usually reached 24 hours after administration and normal

TABLE IV
Anticoagulant Activity of Esters, Ethers, and Acetals of 4-Hydroxycoumarins

Compound		Relative anticoagulant index	Dosage for maximum index
			mg
45	3,3 Methylenebis(4-hydroxycoumarin) diacetate	2.5	50
46	" dipropionate	0.8	50
47	" di-n-butyrate	1.7	50
48	" disobutyrate	5.0	50
49	" di n-valerate	0.8	500
50	" diisovalerate	0.1	500
51	" dicaproate	0.4	200
52	" diheptanoate	Inactive at	500
53	" ditrimethylac- etate	" "	500
54	3,3 Methylenebis(4 hydroxycoumarin) dibenzoate	0.1	500
55	" disalicylate	1.5	50
56	" diacetylsalicyl- ate	Inactive at	500
57	3,3-Methylenebis(4 hydroxycoumarin) monodimethyl- phosphate	1.5	200
58	3,3-Methylenebis(4 hydroxycoumarin) monomethyl ether	5.3	500
59	3,3 Methylenebis(4 hydroxycoumarin) dimethylether	1.1	200
60	3 Phenyl-4-hydroxycoumarin acetylsalicylate	0.6	200
61	3-Phenyl-4-methoxycoumarin	Inactive at	500
62	3-(α Phenyl β acetylethyl)-4-methoxycoumarin	3.8	50
63	2-Methyl-2-methoxy-4 phenyl 5 oxodihydropyrano (3,2 c)(1)benzopyran	60	5
64	2 Methyl 2 ethoxy-4 phenyl-5 oxodihydropyrano (3,2- c)(1)benzopyran	35	5
65	2-Methyl-2 methoxy-4-(p methoxyphenyl)-5 oxodihy- dropyrano(3,2 c)(1)benzopyran	40	5
66	2-Methyl-2 methoxy-4-(p hydroxy m-methoxyphenyl)- 5 oxodihydropyrano(3,2 c)(1)benzopyran	6.0	5

prothrombin times are ordinarily restored within 72 hours, at which time
the bis-4-hydroxycoumarins generally show their maximum effect. The
other active compounds in this group, 33 and 40 to 44, also have their
maximum effect at 72 hours.

A noteworthy compound in this group which exhibits a relatively high activity, 1/15 that of 1, is 3-[6-oxo(1)benzopyrano(4,3-b)(1)benzopyran-7-yl]-4-hydroxycoumarin, 33. The large side group in the 3 position in

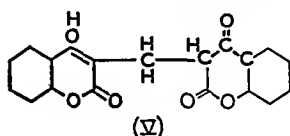
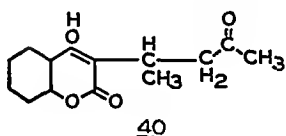


this compound consists of two fused benzopyran rings. The introduction of a hydroxyl into this complex 3-substituent, 34, causes inactivation. That 33 is active by virtue of being a 3-substituted 4-hydroxycoumarin becomes evident by the inactivity of 35 and the very low activity of 36, each of which contains the two fused benzopyran rings of 33 but lacks the 4-hydroxycoumarin residue.

3-Cyano-4-hydroxycoumarin, 32, 3-oximino-4-hydroxycoumarin, 37, 3-nitro-4-hydroxycoumarin, 38, and 3-bromo-4-hydroxycoumarin, 39, are all inactive at the 500 mg level.

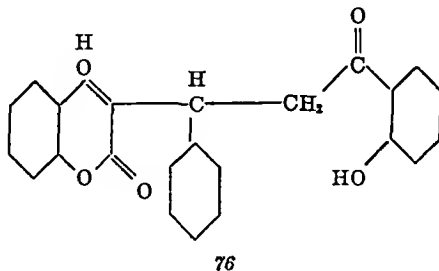
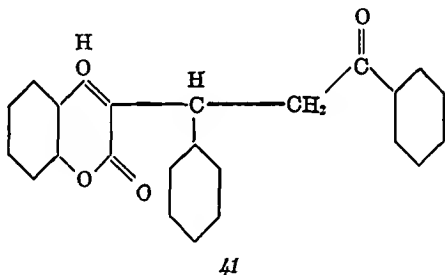
A number of 4-hydroxycoumarins containing a more complex 3-substituent were realized through a Michael type of condensation of 4-hydroxycoumarin with α,β -unsaturated ketones (11). The 3 substituent contains a keto group in a 1,5 arrangement with respect to the 4-hydroxy group. These compounds typified by 40, as indicated by the parts of the formula in bold-faced type, have a configuration in common with the monoketo

tautomer of 1 (V) The potency of this class of compounds approaches that of 1 Compound 40 shows 1/100 and 41, 1/15 of the activity of 1



Several compounds analogous to 3-(α -phenyl- β -acetylmethyl)-4-hydroxycoumarin, 42, were tested The activity of 42 is $\frac{1}{2}$ that of 1 Compound 43 with a methoxyl substituted on the α -phenyl group is $\frac{1}{2}$ as active as 1 When a methoxyl group and a hydroxyl group are present as substituents, 44, the activity is $\frac{1}{8}$ that of 1

3-(α -Phenyl- β -salicylmethyl)-4-hydroxycoumarin, 76, derived from 3,3'-benzylidenebis(4-hydroxycoumarin), 9, by decarboxylation, exhibits 1/100 the activity of 1, while 9 is 1/300 as active as 1 Thus in 9 a phenyl group



on the α -carbon to the 4-hydroxycoumarin moiety profoundly diminishes the activity, while a phenyl group situated in the same position in these condensation products of the Michael type does not reduce the activity This may be accounted for by the fact that 9 may be readily detoxified by anhydride formation to 90 Anhydride formation of the Michael type of

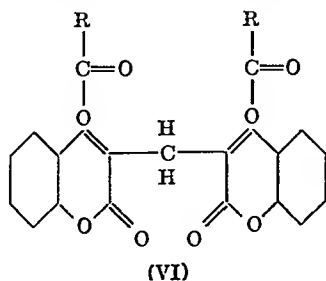
condensation products has not been realized *in vitro*, so it is conceivable that they could not be detoxified readily *in vivo* by this route

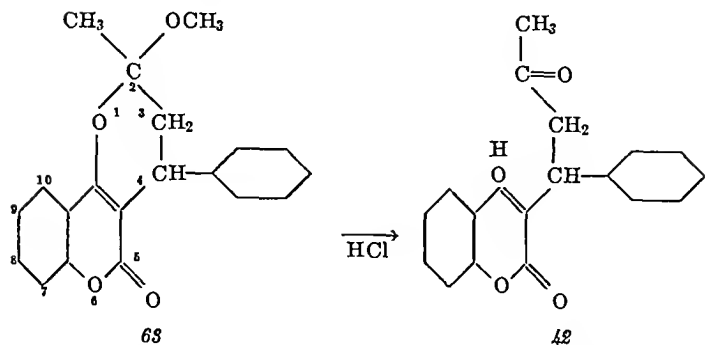
Esters, Ethers, and Acetals of 4-Hydroxycoumarins—Among the diesters (Table IV) of **1** (VI), the acetate **45**, propionate **46**, *n*-butyrate **47**, isobutyrate **48**, and salicylate **55** are the most potent, showing about 1/30 the activity of the parent substance. Diesters of the higher straight chain aliphatic acids show progressively less potency as the chain length is increased. The diheptanoate of **1**, **52**, is inactive. Esters of branched chain acids are less effective than the corresponding straight chain esters. The highly branched ditrimethylacetate, **53**, is inactive at 500 mg.

Among the ethers, the dimethyl ether of **1** is about 1/100 as active as the parent substance. At low levels, as previously reported (1), this derivative is inactive, the 200 mg level being the lowest at which activity is manifested. The monomethyl ether of **1**, **58**, shows 1/20 the activity of the parent substance.

The reduced potencies of the diesters and diethers of **1** and the greater activity of the monomethyl ether, **58**, over the dimethyl ether, **59**, indicate that the enolic hydroxyl groups are involved in producing the anticoagulant effect. The increased lag period before the esters and ethers show their maximum action and the inactivity of the branched chain and higher esters suggest that these compounds act as anticoagulants after conversion by hydrolysis *in vivo* to the parent substances. Chemical studies indicate that the disalicylate of **1**, one of the more active esters, is very readily hydrolyzed (10). This ease of hydrolysis is the probable cause of the large difference in activity between the structurally similar disalicylate, **55**, and dibenzoate, **54**, of **1**.

Among the cyclic acetals of the Michael type of condensation products, **65** shows $\frac{4}{5}$ and **66**, $\frac{1}{2}$ the activity of the straight chain parent compounds **43** and **44**. In contrast **63**, the cyclic methyl acetal of **42**, is about 3 times as active as **42**. It is to be noted that **63**, the cyclic acetal of 3-(α -phenyl- β -acetyethyl)-4-hydroxycoumarin, **42**, is $\frac{2}{3}$ as active as **1**, and rates second in activity among the compounds included in this study. The increased activity of **63** over **42** can be rationalized in several ways. Compound **63**



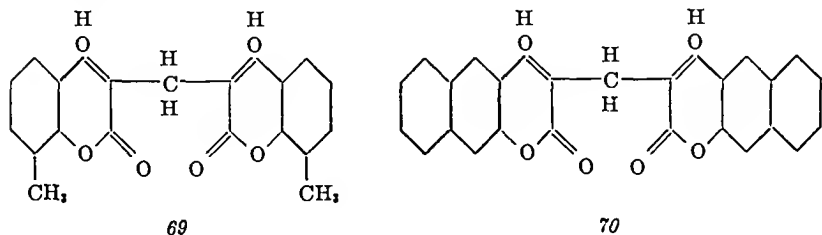


may possess this high activity *per se*, or 63 after a more efficient adsorption than 42 is hydrolyzed to the fairly potent parent *In vitro*, these cyclic acetals can be readily hydrolyzed with acids to their parent products (11)

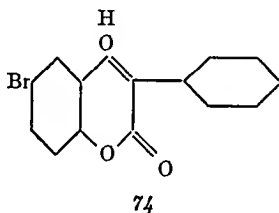
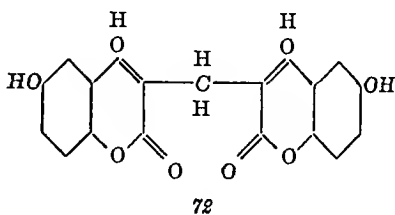
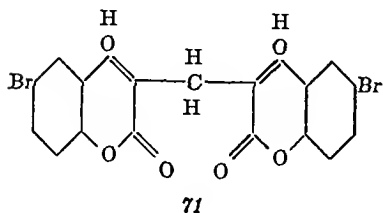
TABLE V
Anticoagulant Activity of Benzenoid Substituted 4-Hydroxycoumarins

Compound	Relative anticoagulant index	Dosage for maximum index
		mg
67 3,3'-Methylenebis(4-hydroxy-6-methylcoumarin)	Inactive at	200
68 3,3'-Methylenebis(4-hydroxy-7-methylcoumarin)	" "	200
69 3,3'-Methylenebis(4-hydroxy-8 methylcoumarin)	1 1	200
70 3,3'-Methylenebis(4-hydroxy-6,7-benzocoumarin)	Inactive at	200
71 3,3'-Methylenebis(4-hydroxy-6 bromocoumarin)	" "	200
72 3,3'-Methylenebis(4,6 dihydroxycoumarin)	" "	200
73 3,3'-Methylenebis(4,7-dihydroxycoumarin)	" "	200
74 3-Phenyl-4 hydroxy-6 bromocoumarin	0 9	500
75 4-Hydroxy 6-methylcoumarin	Inactive at	200

Benzenoid-Substituted 4-Hydroxycoumarins—Of the benzenoid-substituted methyl homologues of 1 only 69 shows slight activity (Table V) Fusion of another benzene ring to the 4-hydroxycoumarin residue, 70, results in inactivation



Introduction of a hydroxyl in the benzene rings of *1* as illustrated by *72* destroys the activity, *73* is also inactive. The 6-bromo derivative, *71*, is inactive, but in contrast 3-phenyl-4-hydroxy-6-bromocoumarin, *74*, shows 1/100 the activity of *1*. The lack of significant potency in this series indi-



icates clearly that this slight modification in the structure of *1* destroys the anticoagulant activity. This effect by benzenoid substitution of hydroxyl, benzo, and methyl groups on *1* is reminiscent of the inactivation of the anti-hemorrhagic properties of 2-methyl-1,4-naphthoquinone (*14*) by analogous structural changes.

Degradation Products of 3,3'-Methylenebis(4-hydroxycoumarin) and Related Compounds—Anticoagulant activity in the 4-hydroxycoumarin group is restricted to *in vivo* action. Furthermore a lag period of from 12 to 24 hours exists before the activity becomes detectable. One possible explanation for this is that activity is dependent on biochemical change in the animal body (*15*). Hence the products realized from certain bis-4-hydroxycoumarins and 4-hydroxycoumarin by chemical degradation *in vitro* were tested.

When the bis-4-hydroxycoumarins are treated with alkali (*16*), the lactone rings are opened stepwise and the resulting β -keto acid is decarboxylated first to products of the type represented by *76* and then to 1,5-diketones like *79* (Table VI). Further degradation leads to salicylic acid, *80* (*15*). All of these products were inactive in the rabbits used at levels up to 500 mg. Compound *76*, which contains one intact 4-hydroxycoumarin residue, showed 1/100 the activity of *1*. The 1,5-diketones from biscoumarins *77*, *78*, and *79*, representing the next stage of degradation, are inactive and *o*-hydroxyacetophenone, *82*, obtained by a comparable degradation of 4-hydroxycoumarin, is inactive. Other diketones, *83* and *84*, and *o*-

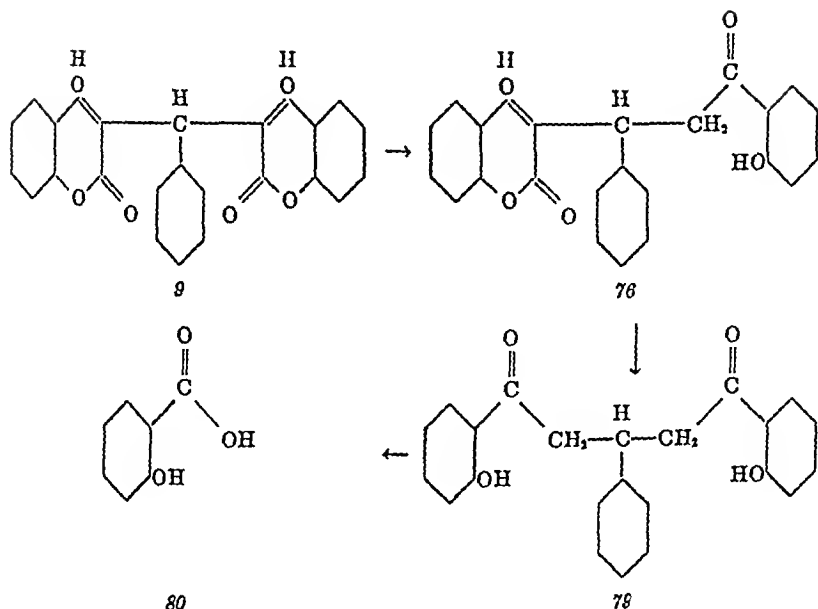


TABLE VI

Anticoagulant Activity of Degradation Products of 3,3'-Methylenebis-(4-hydroxycoumarin) and Related Compounds

Compound	Relative anticoagulant index	Dosage for maximum index
		mg
76 3 (α-Phenyl-β salicylylethyl)-4-hydroxycoumarin	1 1	200
77 1,3 Disalicylylpropane	Inactive at	500
78 1,3 Disalicylyl-2-methylpropane	" "	500
79 1,3-Disalicylyl-2-phenylpropane	" "	500
80 Salicylic acid	" "	500
81 Methylenebis(salicylic acid)	" "	500
82 o-Hydroxyacetophenone	" "	500
83 1,3-Dibenzoylpropane	" "	500
84 Dibenzoylmethane	" "	500
85 Benzal-o-hydroxyacetophenone	" "	500
86 o-Hydroxybenzal-o-hydroxyacetophenone	" "	500
87 Benzalacetophenone	" "	500
88 3,3'-Methylene-4,4'-epoxydicoumarin	" "	500
89 3,3'-Ethylidene-4,4'-epoxydicoumarin	" "	500
90 3,3'-Benzylidene-4,4'-epoxydicoumarin	" "	500

hydroxyketones, 85 and 86, showed no activity at the 500 mg level. Certain α,β -unsaturated ketones, 85 and 87, that gave active products when condensed with 4-hydroxycoumarin via the Michael type of reaction are inactive. The significance of salicylic acid, 80, which does not exhibit activity in the rabbit when single doses up to 500 mg are fed, will be discussed later.

The bis-4-hydroxycoumarins can be dehydrated by the removal of a mole of water between the two enolic hydroxyl groups to form substituted 1,4-pyrans (9) of the general structure represented by (III). These anhydrides, 88, 89, and 90, are inactive.

1,5-Dienols and Other Enols and Ketones—The aldehyde condensation products of 4-hydroxycoumarin contain a 1,5-dienol system, tautomeric with the corresponding 1,5-diketone system. Hence a group of 1,5-

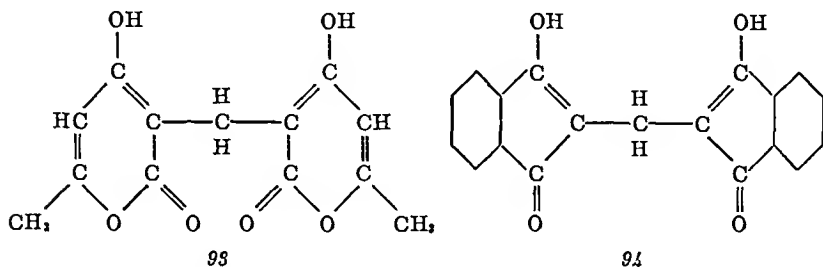
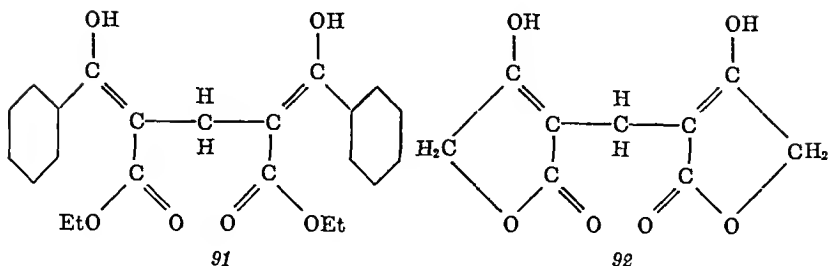
TABLE VII
Anticoagulant Activity of 1,5 Dienols and Certain Enols and Ketones

Compound	Dosage for maximum index (at which anti-coagulant was inactive)
	mg
91 Methylenebis(ethyl benzoylacetate)	200
92 Methylenebis(tetronic acid)	200
93 3,3'-Methylenebis(4-hydroxy-6 methyl α pyrone)	200
94 Methylenebis(indanedione)	500
95 3,3'-Methylenebis(6,6 dimethylidihydroresorcinol)	500
96 Indanedione	200
97 Coumaranone	200
98 Chromanone	200
99 Naphthoresorcinol	500

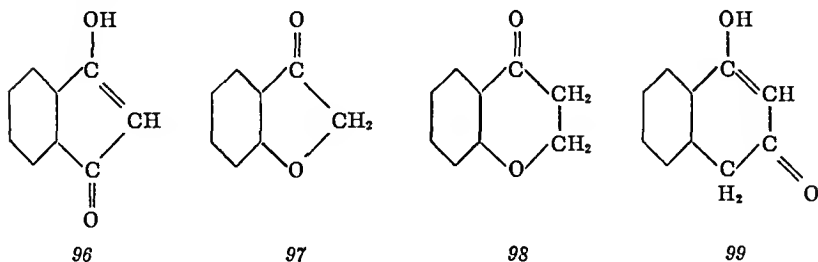
dienols was tested. Because of the activity of the simple 4-hydroxycoumarins, compounds having a keto or enol grouping α to the benzene ring were also examined. The activities of these products are listed in Table VII.

Methylenebis(ethyl benzoylacetate), 91, differs from 1 in that the lactone oxygen ring has been detached from the benzene ring and esterified with ethyl alcohol. Methylenebis(tetronic acid), 92, has a 5-membered lactone ring and lacks the benzenoid group of 1. 3,3'-Methylenebis(4-hydroxy-6 methyl- α -pyrone), 93, has the benzenoid group of 1 replaced by a hydrogen atom and a methyl group. Methylenebis(indanedione), 94, lacks the lactone oxygen of 1.

In spite of the grouping that these 1,5-dienols have in common with 3,3'-methylenebis(4-hydroxycoumarin), they are all inactive.



Certain α -phenyl enols and ketones were tested. Indanedione, 96, lacks the lactone oxygen of 4-hydroxycoumarin. Coumaranone, 97, lacks the carbonyl group of the keto form of 4-hydroxycoumarin. Chromanone, 98, has the carbonyl oxygen of the lactone ring of 4-hydroxycoumarin replaced by hydrogen atoms. Naphthoresorcinol, 99, has the lactone oxygen of 4-hydroxycoumarin replaced by a methylene group. All of these compounds are inactive at the 500 mg level.



The inactivity of the compounds in this class makes it apparent that the 4-hydroxycoumarin structure, a nucleus of fused benzenoid and α -pyrone rings with an enolic hydroxyl on the α -pyrone ring, α to the benzene ring, is necessary for anticoagulant activity.

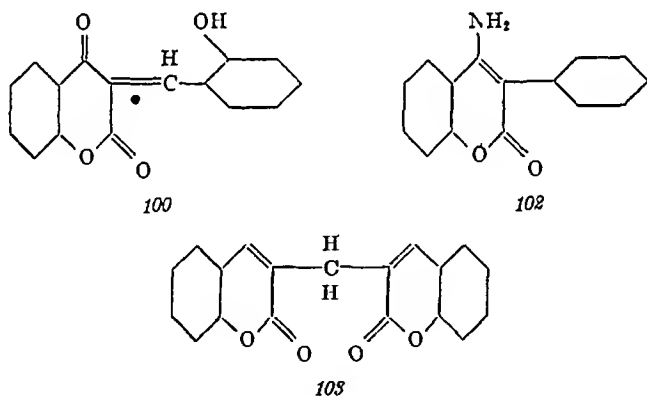
Diketochromans and Coumarins—4-Hydroxycoumarin derivatives have been prepared in which the keto member of the keto-enol pair of tautomers

is stabilized, namely, 100 and 101. They are inactive (Table VIII). When one of these, 3-(*o*-hydroxybenzal)-2,4-diketochroman, 100, is condensed with 4-hydroxycoumarin, the active 3-[6-*o*-(1)benzopyrano(4,3-*b*)(1)benzopyran-7-yl]-4-hydroxycoumarin, 88, is formed. The activity

TABLE VIII
Anticoagulant Activity of Various Diketochromans and Coumarins

Compound	Relative anticoagulant index	Dosage for maximum index
100 3-(<i>o</i> -Hydroxybenzal)-2,4 diketochroman	Inactive at	mg
101 3-(<i>o,p</i> -Dihydroxybenzal)-2,4-diketochroman	" "	200
102 3-Phenyl-4-aminocoumarin	" "	500
103 3,3'-Methylenebis(coumarin)	" "	500
104 3,3'-Carbonylbis(coumarin)	" "	200
105 3,3'-Biscoumarin	" "	200
106 Coumarin	" "	500
	0.02	500

of 88 can be attributed to the fact that it has the 4-hydroxycoumarin structure



Replacement of the enolic hydroxyl of 3-phenyl-4-hydroxycoumarin, 27, which is active, by an amino group, 102, inactivates the molecule. Another indication of the necessity of the enolic hydroxyl group is the inactivity of 3,3'-methylenebis(coumarin), 103. Other biscoumarins, 104 and 105, not containing the 4-hydroxy group showed no activity at the 200 and 500 mg level respectively.

Coumarin, 106, exhibits a barely detectable action at the 500 mg level, the activity being 1/5000 that of 1. The least active compounds in the

4-hydroxycoumarin class, e g 17, 21, 50, and 54, are approximately 5 times more active than coumarin. When 500 mg of coumarin were fed, the increase in prothrombin time over the normal value was 3 seconds. The feeding of three additional 500 mg doses at 12 hour intervals (total 20 gm) did not further increase the prothrombin time. In fact the normal prothrombin values were restored in this interval. The feeding of single massive doses of coumarin (25 gm) usually causes death under the conditions of our bioassay within 24 hours, owing to narcotic action, without visible hemorrhage (17). This is in accord with the findings of Roderick who fed coumarin to rabbits at high levels over a period of days and caused death, but no lesions indicative of hemorrhages were found ((18) p 45). Furthermore, in agricultural practice, large numbers of cattle and other live stock when on sweet clover pasture or on well cured sweet clover hay ingest daily considerable quantities of coumarin,² with apparently no effect on the clotting power of the blood. The hemorrhagic sweet clover disease arises only from the feeding of damaged sweet clover hay or silage which contains 3,3'-methylenebis(4-hydroxycoumarin).

DISCUSSION

The relative hypoprothrombinemia-inducing capacity of 106 compounds in the 4-hydroxycoumarin class or related to it was appraised³. The causative agent of the hemorrhagic sweet clover disease of cattle, 3,3'-methylenebis(4-hydroxycoumarin), 1, is rated the most active of all the compounds studied. This assignment is on the basis of the maximum response per mm of test substance evoked by a single oral dose, with standardized rabbits as the test animal.

Nine 4-hydroxycoumarins showed about 1/10 to 1/2 the activity of 1 at the 5 mg level. The compounds with relatively high potency are of two general types. The most potent bis-4-hydroxycoumarins are the 3,3'-methylene-, 1, 3,3'-ethyldiene-, 2, 3,3'-propyldiene-, 3, 3,3'-butyldiene-, 4, and 3,3'-thiobis(4-hydroxycoumarin), 18. The second type is a 3-substituted 4-hydroxycoumarin with the 3-substituent containing a keto group in the 1,5 position with respect to the 4-hydroxyl group. They are 3-(α -phenyl- β -benzoyl-ethyl)-4-hydroxycoumarin, 41, and 3-(α -phenyl- β -acetyl-ethyl)-4-hydroxycoumarin, 42, and two analogues, 43 and 44, having substituents on the α -phenyl group. The acetals of these compounds also show relatively high activity. The structural interrelationships between the two types have been indicated above.

* Sweet clover hay made from the common varieties of *Melilotus* usually contains 15 to 20 per cent coumarin on the dry weight basis.

³ More than 150 compounds were included in this study.

In general, substitution on the benzenoid ring of these anticoagulants completely destroys the activity. Two minor exceptions showing slight activity are 3,3'-methylenebis(4-hydroxy-8-methylcoumarin), 69, and 3-phenyl-4-hydroxy-6-biomocoumarin, 74.

Fifty of the compounds studied show low activities which are manifested only when the dose is 50, 200, or 500 mg. The activities in this group are from 1/50 to 1/1000 of that exhibited by 1.

The molecular structure necessary for anticoagulant activity, as tested in the rabbit with single doses given orally, is evident from an inspection of the formula. The minimum structural requirements are an intact 4-hydroxycoumarin residue, the 3 position being substituted by a carbon residue or a hydrogen atom. Every compound fulfilling these requirements is active.

For high activity a bis-4-hydroxycoumarin structure or a related type structure (the Michael type of condensation products) having the similar 1,5 spatial relationship between the enolic hydroxyl group of 4-hydroxycoumarin and a keto group is specifically required. Any alteration of this structure results in a decrease in activity. Compounds containing only one 4-hydroxycoumarin residue with an alkyl or aryl group in the 3 position (19 to 28) show greatly diminished activities. Any change made in the fused ring system composing this fundamental 4-hydroxycoumarin residue causes a complete loss of activity.

Of the compounds listed here the work by Jansen and Jensen (4) included 1, 2, 19, 20, 77, 92, 93, 94, and 95, that of Lehmann (5) 1, 2, 16, 19, 20, and 45, and Fantl's (6) 1, 2, and 20. The relative potencies assigned by them to these compounds are substantially in agreement with our findings.

We recently indicated that single doses of salicylic acid administered either orally or intravenously to rats maintained on a basal artificial ration low in vitamin K induce a temporary hypoprothrombinemia comparable in all respects to that caused by 3,3'-methylenebis(4-hydroxycoumarin) (15). Furthermore after continued feeding of salicylic acid the hemorrhagic manifestations resembling those of the sweet clover disease in cattle developed, which eventually became fatal (18). The activity of salicylic acid in the rat is approximately 1/25 that of 1. This evidence and the fact that all of the compounds that showed anticoagulant activity contain the salicyl configuration and yield (theoretically and actually) salicylic acid on oxidation *in vivo* suggested that the 4-hydroxycoumarin anticoagulants might be metabolized in the body to salicylic acid and thereby exert their hypoprothrombinemia-inducing properties. Sustaining support for this suggestion has come from the clinical studies of Meyer and Howard (19), Shapiro, Redish, and Campbell (20), and Rapoport, Wing, and Guest (21), who have recently demonstrated that the commonly used salicylates

(sodium salicylate and acetylsalicylic acid) induce hypoprothrombinemia in man ⁴ It has been noted in this laboratory that benzenoid substitution (methyl, hydroxyl, bromo, and benzo groups) generally destroys the anti-coagulant activity of salicylic acid as well as that of the highly potent 3,3'-methylenebis(4-hydroxycoumarin)

The inactivity of relatively high single oral doses of salicylic acid in the rabbit and dog (species in which avitaminosis K cannot be induced readily (15)) and other issues do not permit an unqualified acceptance of the salicylic acid thesis at present As yet the experimental facts through which it can be definitely affirmed or refuted have not come to hand, but work continues in this laboratory designed to answer the question

SUMMARY

1 On the basis of the hypoprothrombinemia evoked by a single oral dose with standardized rabbits as the test animal, 3,3'-methylenebis(4-hydroxycoumarin) is rated the most potent anticoagulant of the 4-hydroxycoumarin class

2 The minimum structural requirements for activity are an intact 4-hydroxycoumarin residue, with the 3 position substituted by a carbon residue or a hydrogen atom

3 For high anticoagulant potency the bis-4-hydroxycoumarin molecule or a 4-hydroxycoumarin with the 3-substituent containing a keto group in the 1,5 position with respect to the 4-hydroxyl group is necessary

The bulk of the 4-hydroxycoumarin used in this study was kindly supplied by the Abbott Laboratories, North Chicago, Illinois, through Messrs E H Volwiler and Carl Nielsen and Eli Lilly and Company, Indianapolis, Indiana, through Messrs H W Rhodehamel and J P Scott

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REVERSIBLE INACTIVATION OF ADENOSINE-TRIPHOSPHATASE

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In the course of a study of the adenosinetriphosphatase activity of native myosin, it was observed that myosin preparations from which samples were regularly removed showed a continuous decrease in activity. On addition of cysteine or glutathione, enzymatic activity was restored. To determine whether oxidation played a part in this loss of activity, native myosin solutions were treated with dilute hydrogen peroxide in the cold. As a result, adenosinetriphosphatase activity was greatly reduced. The oxidized enzyme could be reactivated by cysteine, glutathione, and, to a lesser extent, ascorbic acid. Similar reactivation of adenosinetriphosphatase by glutathione, following treatment of myosin with potassium chloromercuribenzoic acid, has recently been reported by Barron and Singer (1).

EXPERIMENTAL

Myosin was prepared from the leg muscles of rabbits according to Greenstein and Edsall (2). The monobarium salt of adenosine triphosphate (ATP) was prepared by the method of Kerr (3). The easily hydrolyzable P content was 67.6 per cent of the total P. The barium salt was converted to the sodium salt by treatment with sodium sulfate. The procedure used in measuring adenosinetriphosphatase activity is similar to the one previously described by the author (4). Determinations were carried out in Sorensen's borate buffer at pH 8.6 (5). Inorganic phosphate was determined colorimetrically by the method of Fiske and Subbarow (6). Because of the instability of adenosinetriphosphatase at room temperature in the absence of ATP (7), it was necessary to add the various reagents investigated to cold solutions of myosin. Following addition of ATP, enzymatic hydrolysis was carried out at 37°.

Activation of Stored Myosin—The ability of *l*-cysteine (used as the hydrochloride), glutathione, and *l*-ascorbic acid to restore the adenosinetriphosphatase activity of stored myosin solutions¹ is described in Table I.

¹ The volume of Sample 1 (Table I) was 10 cc. and that of Sample 2 was 150 cc. They were kept at 4° in a refrigerator and contained a trace of toluene added as preservative. Each was opened frequently for the removal of samples. It was found that solutions of relatively smaller volume showed more rapid decreases in activity. It was also observed that the contents of flasks which were not opened for

These substances in 0.1 cc of buffered solutions were added to 1.9 cc of myosin (0.12 mg of N per cc) in chilled borate buffer of pH 8.6. After incubation for 20 minutes in an ice bath under nitrogen, 1 cc of ATP (containing the equivalent of 1 mg of the barium salt) was added and hydrolysis was allowed to proceed at 37° for 20 minutes. 2 cc of 10 per cent trichloroacetic acid were then added, the protein precipitate was removed by centrifugation, and inorganic phosphate was determined. Appropriate control experiments were carried out (see Table I).

TABLE I

Effect of Reducing Substances on Adenosinetriphosphatase Activity of Myosin Corrected for non enzymatic hydrolysis

Sample No *	Age of preparation	P liberated	Reducing agent		P liberated
	days	γ		mg	γ
1	2	18	Cysteine	15	30
	3	16	"	15	30
	8	8	Ascorbic acid	10	14
2	23	13	Glutathione	5	28

* See foot-note 1

TABLE II

Reversal by Reducing Substances of Inactivation of Adenosinetriphosphatase by Hydrogen Peroxide
Corrected for non enzymatic hydrolysis

Enzyme system	P liberated
	γ
ATP + myosin	20
" + " + glutathione	32
" + " + H_2O_2	6
" + " + " + glutathione	27
" + " + " + cysteine	30
" + " + " + ascorbic acid	10

Oxidation of Adenosinetriphosphatase—The inactivation of adenosinetriphosphatase on aging suggested investigation of the influence of mild oxidation on the enzymatic activity and the possibility of reversing the oxidation by means of reducing substances. To 1.8 cc of a cold buffered myosin solution (0.12 mg of N per cc), 0.1 cc of 1 per cent hydrogen peroxide was added. The mixture was allowed to stand for 20 minutes in the

long periods of time showed only small decreases in adenosinetriphosphatase activity which were not reversible by cysteine or glutathione.

cold, and 0.1 cc of catalase² solution (0.15 mg per cc) was then added. The tubes were shaken in the cold for 15 minutes to decompose unchanged hydrogen peroxide. The reducing solution (see Table II) was then added in a volume of 0.1 cc, and, after 15 minutes in an ice bath, 1 cc of adenosinetriphosphatase solution was added. Enzymatic hydrolysis was then allowed to proceed at 37° for 25 minutes.

When myosin was oxidized by 0.05 per cent iodine in 1 per cent potassium iodide solution in the manner described above for hydrogen peroxide, the adenosinetriphosphatase activity was reduced 71 per cent.

Effect of Guanidinium Ion—In view of the finding of Greenstein and Edsall (2) that guanidine salts in low concentration caused the disappearance of the birefringence of flow of myosin, it was of interest to determine the effect of similar treatment on the adenosinetriphosphatase activity. According to the procedure described, 0.2 cc of guanidine solution was incubated with 1.8 cc of buffered myosin in the cold. It was observed that in the presence of 0.15 M guanidine hydrochloride the adenosinetriphosphatase activity as per cent of the control was 57, with 0.30 M guanidine salt, it was 24 per cent.

DISCUSSION

Greenstein and Edsall have observed that the —SH content of native myosin solutions slowly diminishes on standing. The cysteine equivalent of one of their preparations fell from 0.41 to 0.30 per cent in 2 weeks at 4°. In view of the activation of stored adenosinetriphosphatase by cysteine and glutathione, it would appear that decreasing enzymatic activity might be attributed to a fall in —SH content. Activation by sulfhydryl-containing substances has long been known in the case of the "papainases" (8), certain dehydrogenases (9), and other enzymes (1).

Needham (10) has found that iodoacetic acid does not inhibit the activity of adenosinetriphosphatase, and has concluded that the sulfhydryl group is not essential for activity. Her observations may be reconciled with those here reported if one assumes that iodoacetic acid merely blocks the —SH group without producing cross-linkages, whereas inactivation results from the formation of —SS— bridges. The latter change might render inaccessible to phosphorylation (11) those functional groups on the enzyme which are involved in catalysis. Dixon (9) has called attention to the lack of parallel between the effects of oxidized glutathione and iodoacetic acid on the —SH content of enzymes. In explaining the effects of glutathione and cysteine, one must also consider the possibility of direct combination of these substances with the enzyme, as has been reported by Lohmann for glutathione and methylglutathione (12).

The experiments here reported, in which hydrogen peroxide was followed

This was horse liver catalase obtained from Dr. Kurt G. Stern.

by cysteine and glutathione, demonstrate reversal by sulfhydryl-containing substances of the oxidation of adenosinetriphosphatase. Following treatment with hydrogen peroxide, the activity of the enzyme fell 65 to 75 per cent. Cysteine and glutathione restored full activity. Ascorbic acid was relatively weak in this respect, however. Because of the known instability of adenosinetriphosphatase in the absence of ATP, the incubations with hydrogen peroxide and activating agents were carried out in an ice bath. Following the incubation with hydrogen peroxide, catalase was added to destroy unchanged peroxide and thus prevent interference in the subsequent hydrolysis at 37°.

It is difficult to evaluate a possible biological rôle of reversible inactivation of adenosinetriphosphatase. It would appear that this enzyme, in the oxidized state, loses its ability to catalyze the interaction between ATP and the muscle fibril. It has been suggested (11, 13) that reaction with ATP induces relaxation of muscle fiber.

One might conceive that myosin in relaxed muscle is maintained in the phosphorylated (extended) state as a result of a continuous process of phosphorylation and dephosphorylation which maintains a fairly constant number of phosphate groups on the myosin fiber at any given time. Oxidation and reduction of the adenosinetriphosphatase would be expected, from the results here reported, to determine the rate of phosphorylation of the myosin. Thus, the extent of phosphorylation of the myosin fibril and, consequently, its state of contraction would be dependent upon the oxidation-reduction potential of the cell.

As has been reported in the study of papain (8), there appeared to be two types of inactivation of adenosinetriphosphatase on standing. In addition to the formation of an inactive but activatable form, there occurred a progressive irreversible loss in activity. A 2 month-old solution which had lost most of its activity and which had not been exposed to air for 4 weeks could not be activated significantly by glutathione, although calcium ion produced a 30 per cent increase in activity.

The inhibiting effect of low concentrations of guanidine salt (0.15 M) is of interest, because it is in this range of guanidine concentration that myosin loses its birefringence of flow (2). Study of the effects of denaturants on adenosinetriphosphatase may be helpful in establishing whether adenosinetriphosphatase and myosin are identical. The inhibiting effect of guanidine is also of interest in connection with its marked contractile reactions on muscle and its beneficial effects in the treatment of myasthenia (14).

SUMMARY

When native myosin is stored, there is a decrease in adenosinetriphosphatase activity which is in part reversible by cysteine and glutathione.

Adenosinetriphosphatase is inactivated by oxidation with dilute hydrogen peroxide in the cold, and subsequently restored to activity by cysteine or glutathione. Ascorbic acid effects only partial reactivation. Guanidine salts in 0.15 M concentration inhibit adenosinetriphosphatase.

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DENATURATION OF THE GONADOTROPINS BY UREA

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Attempts to correlate the differences in physiologic response to the gonadotropins in terms of structural differences, according to the concept of atomic linkage, have been unsuccessful (1). Chemical studies have invariably brought up the problem of denaturation, with the subsequent changes in secondary valence effects. While it is known that the gonadotropins are subject to denaturation, a comparison of a denaturation under standardized conditions of time, temperature, and concentration¹ has not been undertaken.

The present report is a study of the influence of 40 per cent (by weight) aqueous urea solution on the inactivation of pregnant mare serum, human chorionic, and sheep pituitary gonadotropins at $37.5^{\circ} \pm 0.1^{\circ}$ and pH 7.2 ± 0.2 .

EXPERIMENTAL

Hormones and Assay Procedures—The sheep pituitary and chorionic gonadotropins were stable powders prepared in this laboratory by fractional alcohol precipitation. It was originally planned to precipitate the sheep gonadotropin from the diluted urea solution with tannic acid after the reaction period and use the tannate for the bioassay. It was found, however, that tannic acid did not precipitate the gonadotropin quantitatively from a 5 per cent urea solution. The gonadotropin was precipitated quantitatively by copper from a 5 per cent urea solution at pH 8.5 (see the control in Table II). The assay was performed by using 0.5 mg. of copper as copper sulfate per rat per dosage level. The reference assay curve is given in Table I. One experiment is given in which the assay was performed by measuring the degree of antagonism (3). The chorionic gonadotropin assayed approximately 100 IU per mg. Uterine weight served as the objective measure for the assay of the reaction product (4). Eight to ten rats were used per dosage level in the assays for the pituitary and chorionic gonadotropins and an equal number of litter mates was used for a simultaneous assay of untreated material which was subjected to the same

¹ It is equally true that concentration has not been considered in chemical studies, so that various deductions (2) must be based on the assumption that reactions with ketene, nitrous acid, etc., are of the first order.

conditions of temperature and pH in isotonic solution. The measure of probable error was calculated in the usual manner from the standard deviation of the means of the control and experimental material. Since, however, there is a degree of correlation in the response to litter mates, the

TABLE I
Standard Assay Curve for Sheep Gonadotropin

Dosage level	Mean ovarian weight	Mean uterine weight
mg	mg	mg
1 00	98 ± 7	
0 50	42 ± 3	
0 33	24 ± 2	
0 25	17 ± 1 6	111 ± 6
0 10	12 ± 0 8	99 ± 3
0 05	12 ± 1 0	29 ± 2
0 0	12 ± 0 8	16 ± 1

TABLE II
Inactivation of Sheep Pituitary Gonadotropin to 40 Per Cent Aqueous Urea Solution at 37 5°

Concentration in 40 per cent urea solution	Ratio of control to experimental dose	Mean* organ weight		Estimated recovery of hormone	Correct assay range for 95 per cent of trials	Time of exposure to 40 per cent urea
		Control dose	Experimental dose			
mg per cc		mg	mg	per cent	per cent	hrs
10	1 0	98 ± 9 (o)	98 ± 11 (o)	100	20	0†
10	1 0	76 ± 6 "	51 ± 3 "	75	14	0 25
30	0 5	48 ± 5 "	42 ± 5 "	47	6	0 67
10	0 5	42 ± 6 "	46 ± 7 "	53	10	1 0
10	1 0†	38 ± 5 "	58 ± 6 "	60	32	1 0
10	0 33	24 ± 2 "	22 ± 3 "	32	6	2 0
20	0 33	24 ± 2 "	19 ± 3 "	27	6	2 0
10	0 25	111 ± 6 (u)	68 ± 5 (u)			
		17 ± 1 6 (o)	14 ± 1 0 (o)	7	1 5	6 0

o indicates assay performed by measuring ovarian weight, u by measuring uterine weight

* Mean plus the standard deviation of the mean

† Control for the presence of urea in precipitation by copper

‡ Assay performed by measuring the antagonism

recovery was also calculated for each litter mate in terms of the control. The standard deviation of the mean of the latter figures was used in estimating the recovery as given in Tables II and III. In 50 pairs of rats used in the assay of the sheep gonadotropin, the latter method gave a deviation of 19 per cent 95 per cent of the time in contrast to 25 per cent for the former

method The data for 90 pairs of litter mates used in the assay of the chorionic gonadotropin indicate that when the assay is calculated on the basis of random sampling the value obtained is within 25 per cent of the correct value 95 per cent of the time It is within 21 per cent of the correct value on the basis of litter mate comparison

The hormone from mare serum was the highly purified clinical preparation of the Cutter Laboratories, which is devoid of serum proteins The

TABLE III
Inactivation of Chorionic Gonadotropin (Prolan) by Exposure to 40 Per Cent Aqueous Urea Solution at 37.5°

Pretreatment of prolan*	Concentration of prolan in 40 per cent urea solution	Ratio of control to experimental dose	Mean† uterine weight		Estimated recovery of hormone	Correct assay range for 95 per cent of trials	Time of exposure to 40 per cent urea
			Control dose	Experimental dose			
	IU per gm H ₂ O		mg	mg	per cent	per cent	hrs
A	None	1.0	85 ± 6	89 ± 8	104	±20	0†
B-1	150	1.0	81 ± 7	61 ± 9	80	±20	0.08
A	240	0.5	72 ± 9	89 ± 6	61	±14	0.25
B-2	138	1.0	77 ± 5	25 ± 2.7	42	±7	0.25
B-3	78	0.5	28 ± 3	23 ± 1.2§	43	±8	0.25
A	240	0.5	86 ± 6	60 ± 6	38	±5.0	0.5
"	240	0.4	81 ± 9	31 ± 6	21	±6	1.0
"	240	0.4	81 ± 9	38 ± 7	24	±6	1.0
"	720	0.2	85 ± 8	82 ± 7	20	±4	1.0
"	240	0.1	79 ± 7	93 ± 5	12	±3	2.0
"	240	0.1	81 ± 6	62 ± 9	8	±1.4	6.0
"	720	0.13	88 ± 8	39 ± 5	8	±2.0	6.0
"	240	0.1	77 ± 8	17 ± 0.5	<4.5	Assured	24.0
"	1810	0.025	26 ± 4	42 ± 7	4.0	±0.5	24.0

* A, dry powder, assaying 100 IU per mg, B-1, aqueous solution maintained at room temperature for 15 days (63 per cent of original activity), B-2, for 24 days (57 per cent of original activity), and B-3, for 105 days (32 per cent of original activity)

† Mean plus the standard deviation of the mean

‡ Control for the effect of 3.3 per cent urea concentration at 10°

§ Assay repeated at a higher level with similar results

|| Copper added to delay resorption

assay procedure based on increase in ovarian weight has been previously discussed (1) The recovery, given in Table IV, is calculated on the basis of response in litter mates Eight rats were used per assay dosage level

Results

Order of Reaction—It will be noted that in the experiments in which intact chorionic gonadotropin was employed tripling the concentration

failed to influence the rate of decomposition (see Table III, data for 1 hour and 6 hours) This test, generally conceded to be the best criterion for establishing the order of reaction, places the inactivation of the hormone by urea in the first order It is readily discernible, however, that the progressive inactivation with time does not follow the course of a first order reaction At the 6th hour considerably more activity survives than the theoretical calculation, based on the 15 and 60 minute data, requires This

TABLE IV

Inactivation of Mare Serum Hormone to 40 Per Cent Aqueous Urea Solution at 37.5°*

Mean ovarian weight		Estimated recovery of hormone	Correct assay range for 95 per cent of trials	Time of exposure to 40 per cent urea
Control dose	Experimental dose			
mg	mg	per cent	per cent	hrs
59 ± 5	57 ± 4	97	±8	0 25
67 ± 3	82 ± 8	111	±9	1 0
126 ± 11	121 ± 10	96	±7	1 0
50 ± 3	45 ± 2	95†	±7	1 0
66 ± 5	62 ± 6	96	±7	2 0
63 ± 3	36 ± 2	72	±6	6 0
63 ± 4	23 ± 2	37	±18	22 5

* Reaction concentration of 250 Cole units per cc

† Mean plus the standard deviation of the mean

‡ A commercial preparation of hormone, in aqueous solution for 3 years

TABLE V
Velocity Constant

Time	k (corrected)	k (apparent)
min		
15	0 036 ± 0 009	0 032
30	0 037 ± 0 008	0 033
60	0 033 ± 0 004	0 026
120	0 027 ± 0 003	0 018
360		0 007
1440		0 002

discrepancy may be accounted for on the assumption that in the denaturation the hormone passes over to a form not wholly devoid of activity On the assumption that this transformation is virtually complete the 6th hour and that the new compound is relatively stable to urea, the true concentration of the chorionic gonadotropin = the apparent concentration minus 8/92 of the apparent inactivation With this approximation, the velocity constant k for various time intervals is as shown in Table V The agree-

ment is well within the error of assay. The assumption that the activity of the denatured hormone is relatively stable to urea is borne out by the observation that even after 24 hours exposure of the hormone to urea 50 per cent of the activity found at 6 hours exposure is recovered.

If the inactivation of the pituitary preparation was a second order reaction, tripling the concentration of hormone at 40 minutes should have given a result equal to the result for a unit concentration at 120 minutes. The results (see Table II) do not bear this out. The recovery at 40 minutes, viz 47 ± 3 per cent, is significantly greater than the recovery of 32 ± 3 per cent at 120 minutes. However, it becomes apparent that the reaction is not of the first order since the recovery is affected by concentration.

Comparison of Hormones—The results (see Tables II to IV) clearly demonstrate a marked difference in response of the three gonadotropins to denaturation by urea. 2 hours exposure to 40 per cent urea at 37.5° produced no demonstrable inactivation of the mare serum hormone, while 90 per cent of the activity of the chorionic gonadotropin was destroyed. The rate of inactivation of the sheep pituitary preparation holds an intermediate position. In this connection it is interesting to note that the mare serum hormone, which is known to exhibit a remarkable degree of stability in the organism after administration, in contrast to the two other hormones, is the hormone least sensitive to inactivation by urea denaturation.

The complex nature of the sheep gonadotropic extract as evinced by the antagonism phenomenon and the dual hormone concept is further corroborated by the results of the present study, viz, the dependence of rate of inactivation upon concentration, but failure to establish a reaction order. The excellent agreement of the two assay procedures does not, however, lend support to the dual hormone theory.

Loss of Activity in Aqueous Solution—There are three experiments recorded in Table III in which chorionic gonadotropin, which had suffered an appreciable loss of activity by exposure in aqueous solution at room temperature over the periods of time noted, was subjected to urea denaturation. The product was clearly more sensitive to urea denaturation than the original gonadotropin. These observations demonstrate that the slow inactivation which takes place in aqueous solution at room temperature does not produce the same product which is produced by urea denaturation. If the product was the same, urea should have produced less rather than more inactivation in the experiments cited (see the inactivation rate for the original hormones).

SUMMARY

The rate of inactivation of the physiologic activity of human chorionic, sheep pituitary, and mare serum hormones in 40 per cent urea concentration at 37.5° was studied.

The inactivation of the chorionic gonadotropin proceeded rapidly and was independent of concentration of hormone, but the rate was such that a first order reaction could only be assumed on the basis that the reaction product was a stable substance of less biologic activity. Chorionic gonadotropin partially inactivated by standing in aqueous solution at room temperature was more rapidly inactivated by urea than the original hormone.

The mare serum hormone manifested a remarkable degree of stability.

The inactivation of the sheep pituitary gonadotropin proceeded less rapidly than that of the chorionic gonadotropin and was somewhat dependent upon concentration.

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THE ANTIBACTERIAL ACTION OF DERIVATIVES AND ANALOGUES OF *p*-AMINOBENZOIC ACID

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The bacteriostasis induced by the sulfonamide drugs is reversed by *p*-aminobenzoic acid (PAB) in appropriate concentrations (1). It is now well established that PAB is a normal constituent of cells (2) and that the bacteriostatic effects of the sulfonamides are explicable in terms of their structural similarity to PAB (3). The precise rôle of PAB in cellular metabolism has yet to be clarified but whether it functions as a substrate as some investigators postulate (4), or more likely as a prosthetic group, clearly it must be involved in some reaction which is vital to the growth of many microorganisms. The sulfonamides, presumably by competing with PAB for a part in this key reaction, are able thereby to disrupt the economy of certain growing cells.

Assuming that PAB is the molecule to be imitated, it follows that there must be compounds other than the sulfonamides which can compete with PAB. Thus, for example, sulfur-free compounds related in structure to PAB might be expected to produce bacteriostatic effects comparable to those of the sulfonamides. When the chemotherapeutic possibilities of derivatives and analogues of PAB were investigated, two objectives were in mind: (1) to develop another line of evidence in support of the thesis that the resemblance of the sulfonamides to PAB is the basis of their bacteriostatic properties and (2) to determine whether the bacteriostatic potencies reached by sulfonamides could be duplicated in other molecular configurations and whether the toxic properties which accompany the bacteriostatic effects of the sulfonamides would apply as well to other such bacteriostatic compounds.

When the present investigation was begun, it had been reported that *p*-nitrobenzoic acid arrested quite strongly the growth of certain bacteria (5). Subsequently, Hirsch (6) showed that *p*-aminobenzamide also possessed bacteriostatic properties. While the work here described was in progress, Auhagen (7) and Kuhn and coworkers (8) reported varying degrees of antibacterial action for several sulfur-free compounds related

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in structure to PAB. More recently Wyss and coworkers (9) reported on the biological action of several substituted *p*-aminobenzoic acids. Almost all of the compounds studied in this investigation (cf Table I) had been prepared and tested before the publications mentioned above had appeared or had become accessible. A few, Compounds VII, IX, and XI, were tested after reports of antibacterial action had appeared but were subjected to tests with a greater variety of organisms.

EXPERIMENTAL

The bacteriostatic effects of the various compounds prepared for this investigation were tested on strains of *Escherichia coli*, *Streptococcus hemolyticus* Group A, and *Diplococcus pneumoniae* Type III. A strain of *Escherichia coli* was grown on the Fildes synthetic medium consisting of glucose, asparagine, and inorganic salts. The streptococci were grown on a peptone-beef meat extract broth, while the pneumococci were grown on the same broth enriched with a protein-free filtrate of whole blood of rabbit. Although the above broths contained abundant quantities of anti-sulfonamide substances, the results, being strictly comparative, were not affected by the presence of these substances. Each drug under test was studied over a range of concentrations simultaneously with four sulfonamides, *viz*, sulfanilamide, sulfapyridine, sulfadiazine, and sulfathiazole. The absolute amounts of any drug necessary to inhibit growth varied considerably from one experiment to another, but fortunately all the drugs under test varied in the same way, and the comparative picture remained substantially unchanged.

The tests were carried out in test-tubes of about 1 cm. inside diameter. The inoculum introduced was 0.5 cc. of a 10^{-6} dilution of a young growing culture (6 to 8 hours). The volume of the drug solution was 1.5 cc. or less. The final volume was made up to 5 cc. with sterile medium. The experiments were carried out at 37.5° over a period of at least 72 hours and observations were recorded approximately every 24 hours. The usual + and 0 notation has been employed. Owing to the variability of the results from one run to another, there seemed little advantage to be gained by a more precise estimation of the extent of growth.

Sulfonamide bacteriostasis by definition is reversible by PAB in some suitable concentration. Unless this condition is fulfilled, it is invalid to speak of a sulfonamide-like action. In all the experiments in which growth was arrested reversal by PAB was also checked. When the inhibition of growth was not reversed by PAB, it was assumed that some toxic action was taking place. This was found to be especially true of 4-amino-1-naphthoic acid and 5-nitro-2-furoic acid. Other drugs showed toxic actions particularly at high concentrations. In fact, practically all the drugs which

TABLE I
Benzene Ring Analogues

Compound	M p (corrected)	Calculated per cent			Found per cent		
		C	H	N	C	H	N
Group A Compounds substituted in benzene ring							
	C						
I 3 Methyl- 4 -aminobenzoic acid	173	63 56	6 00		63 62	5 96	
II 3 - Bromo- 4 -aminobenzoic acid	214	38 91	2 80		38 93	2 85	
III 2 Methyl- 4 -aminobenzoic acid hydrochloride	204	51 21	5 37		51 34	5 44	
IV 3 Nitro-4 aminobenzoic acid	292	46 06	3 32		46 19	3 36	
V 2-Acetylamino 4 aminobenzoic acid*	205	55 66	5 19		55 84	5 17	
VI 3 Methoxy- 4 aminobenzoic acid	191	57 48	5 43		57 70	5 30	
VII 2 Chloro 4 -aminobenzoic acid	222	48 99	3 53		49 07	3 90	
VIII 3,5 Dimethyl- 4 -aminobenzoic acid	254	65 44	6 71		65 46	6 91	
Group B Compounds involving variation in carboxyl group							
IX 4,4'-Diaminobenzophenone	245	73 55	5 70		73 53	6 00	
X 2 - (p Aminobenzoylamino) - thiazole*	257-258	54 77	4 14		54 97	4 25	
XI p-Aminoacetophenone	106	71 09	6 71		70 88	6 43	
XII p-Aminophenylarsonic acid†							
XIII p-Aminophenylglycine†							
XIV p Aminophenylacetic acid†							
XV p Aminobenzoyl- l glutamic acid*‡							
XVI p Aminocinnamic acid	174-176	66 24	5 56		66 31	5 77	
Group C Compounds involving variation in amino group							
XVII p-(4 - Diethylaminophenyl azo)-benzoic acid	228-230			14 13			14 01
XVIII p Methylaminobenzoic acid	160	63 56	6 00		63 52	6 29	
XIX p - Dimethylaminobenzoic acid	242	65 44	6 71		65 44	6 90	
XX p - Acetylaminomethylbenzoic acid*	191	62 16	5 74		62 42	5 81	
XXI p Guanidobenzoic acid	296			23 45			23 23

TABLE I—Concluded

Compound	M _p (corrected)	Calculated, per cent			Found, per cent		
		C	H	N	C	H	N
Group D Compounds in which certain combinations of variations of Groups A, B, and C are involved							
	C						
XXII 3 - Methyl- 4 nitrobenzoic acid	218	53 04	3 90		53 14	4 07	
XXIII 3 Methyl-4 aminobenzamide	125	63 98	6 71	18 66	64 32	6 78	18 98
XXIV 2,4-Dinitrobenzoic acid†							
XXV 4,4'-Dinitrobenzil	214	56 00	2 69		56 13	2 87	
XXVI p - Dimethylaminobenzaldehyde†							
Analogues Having Other Ring Systems Than Benzene Ring							
XXVII 4-Amino-1-naphthoic acid	195-200	70 57	4 85	7 48	70 57	5 08	7 65
XXVIII 5 Amino thiophene 2 -carboxamide hydrochloride*		33 61	3 95		33 82	4 10	
XXIX 5 Nitro thiophene- 2 -carboxamide*	191	34 88	2 34		34 76	2 60	
XXX 5 - Nitro thiophene- 2 -carboxylic acid	159	34 68	1 74	8 09	34 97	1 89	7 80
XXXI Methyl- 2 -(5 acetylamino thienyl) ketone*	279	52 44	4 95		52 64	5 24	
XXXII 2-Amino 5 carboxythiazole*	191	33 33	2 80		33 58	2 79	
XXXIII 5 Nitro-2 furoic acid	188	38 23	1 92	8 92	38 34	1 97	8 71
XXXIV 5-Acetylamino-2 furoic acid	240	49 71	4 17		49 66	4 45	
XXXV 6-Amino 3 carboxypyridine	318	52 17	4 38		51 86	4 37	

* These compounds were first prepared in this investigation

† Gift from Dr Michael Heidelberger

‡ Eastman Kodak products

§ Gift from Dr Sarah Ratner

were bacteriostatic at certain concentrations as indicated by reversal in the presence of PAB were bactericidal at higher concentrations, as shown by non-reversal in the presence of PAB

The solubilities of the thirty-five compounds under test varied from a few micrograms per cc to about 50 mg per cc. In some cases it was impossible to get a high enough concentration of the substance in the growth medium for satisfactory tests and the negative bacteriostatic results obtained in these instances need qualification on the basis of the limited solubility.

Results

The bacteriostasis induced by p-nitrobenzoic acid and p-aminobenzamide was more closely examined, since these were the first sulfur-free compounds

reported to behave like the sulfonamides. Experiment showed that neither *p*-nitrobenzoic acid nor *p*-aminobenzamide was active toward all the bacteria inhibited by the sulfonamides. Whereas, for example, they inhibit the growth of *Escherichia coli* (Table II), they have slight if any effect on the growth of *Streptococcus hemolyticus*. Furthermore, even when growth is inhibited as in the case of *E. coli*, the inhibition is short lived, and after 45 hours or more, depending upon the initial concentration of the drug, normal growth takes place. Since these two compounds can be converted to PAB, in one case by hydrolysis, and in the other by reduction, it seemed reasonable to assume that when they were inactive, as in the case of the

TABLE II

Effect of p-Nitrobenzoic Acid, p-Aminobenzamide, and Sulfanilamide on Growth of Escherichia coli and Streptococcus hemolyticus

Drug	γ per cc	<i>E. coli</i>			<i>S. hemolyticus</i>		
		24 hrs	48 hrs	94 hrs	24 hrs	48 hrs	94 hrs
Control	0	++++	++++	++++	++++	++++	++++
<i>p</i> -Nitrobenzoic acid	800	0	0	+	+	++++	++++
	400	0	0	++++	++++	++++	++++
	100	0	0	++++			
	40	0	++++	++++			
	20	+	++++	++++			
<i>p</i> Aminobenzamide	300	0	0	+	0	++++	++++
	270	0	0	+			
	190	0	0	++++			
	150	0	+	++++	0	++++	++++
Sulfanilamide	100	0	0	0	0	0	0
	50	0	0	0	0	0	0
	25	0	+	++++	0	0	++
	5	+	++++	++++	++	++++	++++

S. hemolyticus, they were being transformed rapidly to PAB, and their bacteriostatic effects were thereby obscured. In the same way the short lived bacteriostatic effects on *E. coli* could be explained in terms of a slow conversion of the PAB derivatives to PAB. These conclusions were borne out by the demonstration that both *p*-nitrobenzoic acid and *p*-aminobenzamide were able to reverse completely the bacteriostatic effects of sulfonamide drugs on the growth of *S. hemolyticus* and *E. coli* (Table III).

Thus, at the start of the investigation we realized that it was unwise to prepare compounds which in the presence of bacterial enzymes could be converted to PAB. Our efforts therefore were directed mainly along the lines of biologically stable derivatives and analogues of PAB and amino-carboxylic acids and nitrocarboxylic acids of other ring systems.

Benzene Ring Analogues—The twenty-six compounds in this class may be divided into (Group A) those in which the benzene ring has been substituted, (Group B) those in which the carboxyl group has been replaced or substituted, (Group C) those in which the amino group has been replaced or substituted, and (Group D) those which involve certain combinations of variations of Groups A, B, and C

Only eight of the benzene ring analogues showed bacteriostatic properties, these were Compounds I, II, III, V, VI, and VII of Group A, Compound XI of Group B, and Compound XXIII of Group D (cf Table IV) None of the compounds of Group C was active Among the active ring-substituted analogues the bacteriostatic potency never exceeded one-fifth that of sulfanilamide or about one-one-hundredth that of the most active sulfonamide

TABLE III

Reversal by p-Aminobenzamide and p Nitrobenzoic Acid of Bacteriostasis Induced by Sulfapyridine

	<i>Escherichia coli</i>		<i>Streptococcus hemolyticus</i>	
	24 hrs	42 hrs	24 hrs	72 hrs
Control without drug	+++++	+++++	+++++	+++++
Sulfapyridine (A), 15 γ per cc	0	0	0	0
p Aminobenzamide (B), 125 γ per cc	++	+++++	+	+++++
A + B	++	+++++	+++	+++++
p Nitrobenzoic acid (C), 40 γ per cc	0	+++++	+++++	+++++
A + C	+	+++++	+++++	+++++

The bacteriostatic effects of active analogues of PAB were completely reversed by addition of PAB (Table V) Tests also have been made to determine whether analogues of PAB could replace PAB in reversing the bacteriostatic action of the sulfonamides Thus far only three of the twenty six analogues of PAB have been found to possess any significant anti sulfonamide activity, viz, Compounds VII, XV, and XVI (Table VI) It is of interest that Compound VII, 2-chloro-4-aminobenzoic acid, exhibited strong PAB activity towards *Streptococcus hemolyticus* and *Diplococcus pneumoniae*, whereas in the case of *Escherichia coli* it was weakly bacteriostatic at high concentrations ($>100 \gamma$ per cc) and exhibited antisulfonamide action at lower concentrations ($<20 \gamma$ per cc) The possibility had to be explored that the antisulfonamide actions of Compounds VII, XV, and XVI were referable to traces of PAB formed by degradation or conversion of these substances in the presence of bacteria This point was checked by determining the antisulfonamide activity of these three compounds after incubation with bacteria No increase in activity was ob

TABLE IV
Concentrations for Bacteriostasis

The values are given in micrograms per cc

Compound	Minimum over 64 hr period			Maximum tested
	<i>E coli</i>	<i>S. hemolyticus</i>	<i>D. pneumoniae</i>	
I 3 Methyl 4 aminobenzoic acid	300	300		4000
II 3 Bromo 4-aminobenzoic "	600	600		2000
III 2 Methyl-4 aminobenzoic " hydrochloride	4000			8000
IV 3 Nitro 4 aminobenzoic acid				2000
V 2 Acetyl amino 4-aminobenzoic acid		8000		8000
VI 3-Methoxy-4 aminobenzoic acid	8000	200		8000
VII 2 Chloro-4 aminobenzoic "	200			2000
VIII 3,5 Dimethyl-4 aminobenzoic acid				500
IX 4,4'-Diaminobenzophenone				10
X 2 (p Aminobenzoylamino)-thiazole				1 5
XI p Aminoacetophenone	600	500	400	700
XII p Aminophenylarsonic acid				500
XIII p Aminophenylglycine				2000
XIV p-Aminophenylacetic acid				4000
XV p Aminobenzoyl l-glutamic acid				400
XVI p Aminocinnamic acid				4000
XVII p (4 Diethylaminophenylazo) benzoic acid				400
XVIII p Methylaminobenzoic acid				2000
XIX p Dimethylaminobenzoic acid				200
XX p-Acetylaminomethylbenzoic acid				1000
XXI p Guanidobenzoic acid				500
XXII 3 Methyl 4 nitrobenzoic acid				2000
XXIII 3 Methyl-4 aminobenzamide	2600	1400		3200
XXIV 2,4 Dinitrobenzoic acid				1000
XXV 4,4'-Dinitrobenzil				10
XXVI p Dimethylaminobenzaldehyde				100
XXVII 4-Amino 1-naphthoic acid				400
XXVIII 5 Aminothiophene 2-carboxamide hydrochloride				1000
XXIX 5 Nitrothiophene-2 carboxamide	4	5	40	500
XXX 5-Nitrothiophene 2 carboxylic acid	40	2 5	20	2000
XXXI Methyl 2 (5 acetylaminothiophenyl) ketone	20	40	60	80
XXXII 2 Amino 5 carboxythiazole				2000
XXXIII 5 Nitro 2 furoic acid				4000
XXXIV 5 Acetyl amino 2 furoic acid				2000
XXXV 6 Amino 3 carboxypyridine	5	12		1000
Sulfanilamide	40	12	50	2000
Sulfapyridine	4	10	10	2000
Sulfathiazole	0 4	5	5	2000
Sulfadiazine	0 4	2	2	2000

served It must therefore be assumed that these three analogues of PAB have one-fortieth to one-two-hundredths the antisulfonamide activity of PAB

Naphthalene Ring Analogue—Compound XXVII was bactericidal at high concentrations (10 to 40 mg per cent) and inactive at lower concentrations (<10 mg per cent) Bacteriostasis did not obtain in the intermediary range of concentrations

Thiophene Ring Analogues—Compounds XXIX and XXX were both highly active and compared favorably with the most active sulfonamides,

TABLE V
Reversal by *p* Aminobenzoic Acid of Bacteriostasis Induced by Analogues of *p*-Aminobenzoic Acid

Under test		Concentration		Time for reversal	Under test		Concentration		Time for reversal
Compound No	Organism	Analogue	<i>p</i> Aminobenzoic acid		Compound No	Organism	Analogue	<i>p</i> Aminobenzoic acid	
		γ per cc	γ per cc	hrs			γ per cc	γ per cc	hrs
I	<i>S hemolyticus</i>	300	6	<24	XI	<i>E coli</i>	600	6	<24
"	<i>E coli</i>	300	6	24	"	" "	200	6	40
II	<i>S hemolyticus</i>	1000	6	<15	"	<i>S hemolyticus</i>	600	1	40
"	"	700	1	40	XXIII	" "	2000	6	<16
III	<i>E coli</i>	4000	1	18	"	<i>E coli</i>	2800	6	40
V	<i>S hemolyticus</i>	8000	1	<17	XXIX	<i>D pneumoniae</i>	25	1	40
VI	" "	800	1	24	XXX	<i>S hemolyticus</i>	4	6	<24
"	" "	80	1	20	"	<i>D pneumoniae</i>	70	6	<24
"	<i>E coli</i>	8000	1	64	XXXI	" "	80	1	64
"	" "	2000	1	15	"	" "	80	5	40
VII	" "	200	1	15	XXXV	<i>S hemolyticus</i>	100	1	22
"	" "	100	1	15	"	" "	10	1	20
					"	<i>E coli</i>	5	1	40

at least in their action on *Streptococcus hemolyticus* (Table IV) These bacteriostatic effects were reversed by appropriate concentrations of PAB At high concentrations of these two drugs, the inhibitory effects on growth were not reversed by PAB The zone between bactericidal and bacteriostatic effects was fairly sharp It is interesting to note that the bacteriostatic effects of Compounds XXIX and XXX did not always run parallel Thus, whereas Compound XXIX was about 10 times as active as Compound XXX towards *Escherichia coli*, Compound XXX was twice as active as Compound XXIX towards *Streptococcus hemolyticus* and *Diplococcus pneumoniae*

Thiazole Ring Analogue—Compound XXXII showed no bacteriostatic action on the growth of the three test organisms

Furan Ring Analogues—The two compounds of this group, Nos XXXIII and XXXIV, were found to be inactive. At concentrations of about 100 to 400 mg per cent Compound XXXIII was bactericidal and its inhibition of growth was not reversed by PAB

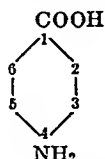
Pyridine Ring Analogues—Compound XXXV showed considerable bacteriostatic activity towards *Escherichia coli* and *Streptococcus hemolyticus* but no appreciable activity towards the pneumococcus (Table IV). The bacteriostatic effects were reversed by PAB

TABLE VI
Antisulfonamide Action of Analogues of *p*-Aminobenzoic Acid

Analogue	Organism	Concentration		Reversal time
		Analogue	Sulfapyridine	
		γ per cc	γ per cc	hrs
VII 2-Chloro-4 amino-benzoic acid	<i>D pneumoniae</i>	2	15	17
	<i>S hemolyticus</i>	20	15	64
	<i>E coli</i>	20	15	24
XV <i>p</i> -Aminobenzoyl-l-glutamic acid	<i>D pneumoniae</i>	0.4	40	64
	<i>S hemolyticus</i>	4	15	64
	<i>E coli</i>	400	15	64
XVI <i>p</i> Aminocinnamic acid	<i>D pneumoniae</i>	1	15	64
	<i>S hemolyticus</i>	1	15	64
	<i>E coli</i>	150	15	64
<i>p</i> -Aminobenzoic acid	<i>D pneumoniae</i>	0.01	15	64
	<i>S hemolyticus</i>	0.05	15	24
		0.10	15	16
	<i>E coli</i>	0.01	15	64

Relation of Structure to *p*-Aminobenzoic Acid Antagonism

Isocyclic Compounds—On the basis of the compounds thus far tested, the following generalizations can be advanced



1. Monosubstitution by neutral or weakly electropositive groups in the 2 or 3 position of PAB yields compounds with bacteriostatic properties. There is little to choose between the two positions as far as activity is concerned.

2 Disubstitution, whether in the 2,3 positions (Compound XXVII) or in the 3,5 positions (Compound VIII), results in compounds which exhibit no bacteriostatic action

3 Replacement of the amino group by any of the groups thus far studied other than nitro results in inactive compounds

4 Variation of the carboxyl group by replacement or by derivative formation may give compounds which exhibit PAB activity, bacteriostatic activity, or neither. No rational conclusions can be drawn

5 Simultaneous variation of the amino group, including replacement by the nitro group, and substitution in the benzene nucleus result in inactive compounds (e.g. Nos XXII and XXIV)

6 Simultaneous variation of the amino and carboxy groups gives inactive compounds (e.g. Nos XXV and XXVI)

Heterocyclic Compounds Thiophene Analogues of PAB—The isostere of *p*-aminobenzamide, 5-aminothiophene-2-carboxamide, was found to be inactive, while the isosteres of *p*-nitrobenzamide and *p*-nitrobenzoic acid were highly active. The inactivity of Compound XXVIII may be due to a variation in chemical activity of the amino group in the thiophene nucleus from that of a normal aromatic amino group. Such a variation may be dependent upon tautomeric effects, oxidation potential, or other properties.

In view of the inactivity of Compound XXVIII, the activity of Compounds XXIX and XXX is difficult to interpret, since the nitro group does not resemble the aromatic amino group either in respect to chemical reactivity or physical dimensions. It seems possible that some reduction product of the nitro group which more closely resembles an aromatic amino group might be formed and be responsible for the bacteriostatic action. Efforts to prepare the isostere of PAB were not successful.

Thiazole Analogue—The inactivity of 2-amino-5-carboxythiazole might be due to the magnitude of the nucleus alteration as compared to PAB or to variations in the chemical properties of one of the functional groups. Attachment of the amino group to a carbon atom linked directly to sulfur is a common property of Compounds XXXIII and XXXII, a fact which is of possible significance.

Furan Analogues—The inactivity of the furan derivatives as bacteriostatic agents suggests that the furan nucleus represents too wide a departure from the phenyl nucleus either for physical or chemical reasons.

Pyridine Analogue—The rather high activity of 6-amino-3-carboxypyridine as contrasted with the complete inactivity of its isostere, 2-amino-5-carboxythiazole, provides further evidence that chemical reactivity of functional groups may be of greater significance than physical dimensions of the molecule in determining antibacterial activity for compounds related in structure to PAB.

The authors desire to express their thanks to Dr R T Major for his interest and guidance in this work. Microanalyses were executed in the Merck Laboratories under the direction of Mr D F Hayman

SUMMARY

Thirty-five compounds related in structure to PAB have been studied to determine their effect upon the growth of three bacteria. Twelve showed bacteriostatic effects which could be reversed by PAB, whereas three compounds behaved like PAB albeit much more weakly in reversing the bacteriostasis induced by the sulfonamides. One of the compounds (2-chloro-4-aminobenzoic acid) showed bacteriostatic effects at high concentrations and PAB action at lower concentrations.

Evidence is presented to indicate that the antibacterial action of PAB analogues may be determined by chemical reactivity of functional groups as well as similarity in physical dimensions of the molecules to PAB.

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GLUCURONIC ACID SYNTHESIS AND THE GLYCOGEN CONTENT OF THE LIVER OF THE RAT*

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It has been known for many years that a number of foreign compounds of varying degrees of toxicity are "detoxicated" by conjugation with glucuronic acid and excreted in the urine as conjugated glucuronates. It is believed that the liver is the chief organ in which the synthesis of glucuronic acid and its conjugation with these toxic substances occurs. The distribution of β -glucuronidase in the mammalian organism has suggested, however, that the process of glucuronic acid conjugation is not entirely confined to the liver (1). Evidence of the rôle of the liver in glucuronic acid metabolism has been obtained in perfusion (2) and tissue slice (3) experiments, by hepatectomy (4), by the use of hepatotoxic substances (5, 6), and in studies of human pathology (7).

The chemical origin of the glucuronic acid used in detoxication is not clear. It is improbable that it arises from preformed glucuronic acid in the organism. Quick (8) has suggested that the acid is derived from carbohydrate and considers it probable that "the precursor of glucuronic acid is derived more readily from glycogen or glycogenic amino acids than from glucose." Such an origin from glycogen rather than from glucose is not inconsistent with the present hypothesis of the metabolism of carbohydrate in which phosphorylation, rather than simple hydrolysis, of glycogen plays an important rôle.

If glycogen serves as the precursor of glucuronic acid, it might be anticipated that administration of compounds for whose metabolism glucuronic acid is mobilized might result in a diminution of liver glycogen. The results here presented show that, after the administration of *l*-menthol or of sodium tertiary butyl acetate to young white rats, the content of the glycogen in the liver is lower than in control animals. *l*-Menthol has long been known to be excreted as menthylglucuronic acid and, in unpublished experiments from this laboratory, the oral administration of the sodium salt of tertiary butylacetic acid has been shown to result in the excretion of considerable amounts of "extra" glucuronic acid.

* This work was supported in part by a grant from the Mallinckrodt Chemical Works of St. Louis.

EXPERIMENTAL

Male white rats, of from 145 to 200 gm in weight, were fed a standard stock diet *ad libitum* for 7 to 14 days, food being available at all times until the experimental compounds were administered by stomach tube. After the beginning of the experimental period, water was available but no food was given. After 4 hours, the rats were killed by a blow on the back of the neck, and the glycogen content of the liver was determined by the method of Good, Kierner, and Somogyi (9).

Four groups of animals were used in parallel experiments. Group 1 received 2 ml of water and served as controls. Group 2 was fed by tube 0.40 to 0.45 gm of *l*-menthol, which had been melted and kept at a temperature of 50–60°. Groups 3 and 4 received 2 ml of solutions containing 0.40 and 0.318 gm, respectively, of the sodium salts of tertiary butylacetic and butyric acids. Group 4 served as an additional control, since butyric acid is readily oxidized and is not known to be excreted in conjugation with glucuronic acid. The amounts of the three experimental substances fed were approximately equimolecular. Since menthol was not soluble in water, it was difficult to administer it quantitatively.

Examination of Table I shows that the glycogen contents of the livers of those rats fed either of the two substances known to increase the excretion of urinary glucuronic acid were definitely less than those of rats which received either water alone or the easily oxidizable sodium butyrate. There was no overlapping of the values in the different groups, except with one rat in the control (water) group, and there is every reason to believe that the results are significant. The high values for liver glycogen observed after the feeding of sodium butyrate demonstrate that the administration of the salt of a fatty acid does not necessarily result in a lowered content of liver glycogen. The significance of the higher concentration of liver glycogen after sodium butyrate as compared with the control group fed water only is not clear. It is possible that the butyrate is oxidized readily and thus protects the liver glycogen so that glycogenolysis is diminished or that under the conditions of these experiments butyric acid contributes to the synthesis of liver glycogen. Although butyric acid is not usually considered as a glycogenic substance, recent experiments have indicated a conversion of butyric acid to glycogen of the liver (10).

It was not possible to determine glucuronic acid in the urine of the particular animals sacrificed for the determination of liver glycogen. Determinations of the glucuronic acid content of the urine of other rats receiving menthol or the salt of tertiary butylacetic acid have shown, as in the rabbit, a very considerable increase in the glucuronic acid content of the urine. No similar increase was observed when sodium butyrate was fed. There appears to be little question, therefore, that the diminished content

of liver glycogen recorded in Table I is to be related to glucuronic acid synthesis in the organism

TABLE I

Glycogen Content of Liver of Young White Rat after Oral Administration of l Menthol and Sodium Salts of Tertiary Butylacetic and Butyric Acids

The animals were killed after a 4 hour absorption period. The amounts of the various compounds fed were approximately equimolar (2.9 mm)

Group No	Substance fed	Body weight	Liver		
			Weight	Glycogen	Average glycogen
		gm	gm	per cent	per cent
1	Water, controls	164	6.75	3.42	3.58
		162	6.40	4.43	
		180	8.80	1.03	
		146	6.83	3.71	
		149	7.10	4.28	
		188	9.80	4.87	
		180	8.00	3.83	
		163	7.60	3.06	
2	l-Menthol	156	5.55	1.74	1.60
		156	6.10	1.42	
		180	8.65	1.30	
		168	6.93	1.77	
		171	6.75	1.70	
		191	9.15	1.36	
		173	7.15	1.95	
		176	7.55	0.60	
3	Sodium tertiary butyl acetate	162	5.90	0.57	0.95
		195	7.90	1.24	
		163	9.05	1.07	
		152	5.55	0.14	
		180	7.00	1.43	
		187	8.70	1.20	
		180	8.40	1.35	
		176	9.20	5.92	
4	Sodium n butyrate	184	9.95	6.05	6.39
		196	9.70	6.95	
		197	9.70	5.00	
		180	10.78	8.01	
		161	9.05	6.81	
		190	10.50	6.10	
		187	9.40	6.35	

Previous suggestions of the relation of liver glycogen to glucuronic acid synthesis have been limited to qualitative and histological observations. Thus Schmid (11) showed that, in hibernating frogs whose liver glycogen

was depleted, the tolerance for menthol was greatly diminished, while Kobayashi (12), in experiments with rabbits, observed that the liver cells contained little glycogen, as detected by histological methods, when the animals had previously received avertin (tribromoethanol) which is excreted as a conjugated glucuronate. Lutwak-Mann (13) found that the livers of rats which received salicylic acid subcutaneously contained little glycogen in 4 to 7 hours after the injection. Although it has been stated that salicylic acid is excreted as a glucuronide, Lutwak-Mann was unable to detect any appreciable amounts of glucuronic acid in the urine, and interpreted the disappearance of liver glycogen as due to the "profound changes in metabolism" resulting from the injection of salicylate, rather than from the utilization of liver glycogen in the synthesis of glucuronic acid. The present experiments suggest strongly a rôle of glycogen in the mobilization and production of glucuronic acid for detoxication.

SUMMARY

4 hours after the oral administration of *l*-menthol or sodium tertiary butyl acetate to well fed young white rats, the glycogen content of the liver was much depleted in comparison to that of control animals which were fed water alone or sodium butyrate. Since the administration of *l*-menthol or tertiary butylacetic acid results in the excretion of considerable amounts of "extra" glucuronic acid in the urine, the data suggest that liver glycogen is used in the synthesis of glucuronic acid.

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BUTYRATE OXIDATION BY LIVER ENZYMES

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It has been found (1) that lower saturated fatty acids are oxidized by a system formed by a preparation of liver enzymes with the addition of adenylic acid, cytochrome *c*, inorganic phosphate, magnesium ions, and fumarate. In the absence of added fatty acids phosphopyruvate or a similar substance accumulated, whereas this did not occur in the presence of fatty acids.

This paper describes experiments which were undertaken in order to study the possibility of replacing fumarate by other components of the citric acid cycle, the formation of phosphopyruvate and its influence on the oxidation of butyrate, and the relation between oxidation products and oxygen uptake.

EXPERIMENTAL

Methods

The general technique was the same as that described previously (1). The concentration of magnesium chloride used for preparing the enzyme was 0.5 M instead of 1 M. Experiments were carried out in 50 cc Erlenmeyer flasks filled with oxygen and shaken at 80 oscillations per minute. It was found that in air the reaction rate may be limited by the rate of oxygen diffusion.

The following methods of preparation were used: adenylic acid, Lohmann (2), phosphopyruvic acid, Kiessling (3), 3-phosphoglyceric acid, Neuberg and Kobel (4), sodium pyruvate, Robertson (5). The sample of sodium α -glycerophosphate was from The British Drug Houses, Ltd. All substances were added as sodium salts.

Methods of estimation were as follows: phosphate, after Fiske and Subbarow (6), butyrate after Muñoz and Leloir (1), ketone bodies after Edson (7), phosphopyruvate after Lohmann and Meyerhof (8) and by estimating pyruvate before and after 1 hour's hydrolysis in 1 N HCl at 100°. A colorimetric method with 2,4-dinitrophenylhydrazine was used (9).

Some Properties of Enzyme System—The activity of the system is maintained for more than 90 minutes at 25° in oxygen when all the components and substrate are present. If butyrate, adenylic acid, or cytochrome is added after 10 to 15 minutes incubation, there occurs a complete inactivation.

tion If pyruvate instead of butyrate is added, the activity is maintained. It appears that the system is more stable when certain substrates are being oxidized. Many unsuccessful attempts have been made to stabilize the system by other procedures.

Microscopic examination shows that the preparation contains nuclei and many cell particles.

The preparation appears yellowish white and a spectroscopic examination shows a band at about 560 $m\mu$, corresponding probably to cytochrome *b*, and a fainter band at about 600 $m\mu$, corresponding to cytochrome *a*. No cytochrome *c* is detectable. As these bands appear on reduction, they can be used in ascertaining the activity of the system.

Action of Different Substances on Butyrate Oxidation—It was previously stated (1) that fumarate could not be replaced by succinate. As is shown

TABLE I

Action of Succinate, Malate, Citrate, and Glutamate on Oxidation of Butyrate

Disappearance of butyrate (micromoles) produced by 2.5 ml of liver enzymes + 0.2 ml of $M/15$ phosphate buffer of pH 7.7 + 0.1 ml of 0.1 M magnesium chloride + 0.5 mg of cytochrome *c* preparation + 1 mg of adenylic acid + 0.1 ml of 0.1 M sodium α -glycerophosphate + 17 micromoles of butyrate. Total volume, 6 ml. 90 minutes at 25° in oxygen.

Additions	200 micromoles	20 micromoles	2 micromoles
Succinate	1.8	8.9	8.5
Fumarate	8.6	9.4	8.4
Malate	0	9.6	8.0
Citrate	1.2	8.3	8.4
Glutamate	6.1	7.4	3.4
None, 2.4, 1.7			

In Table I, this is true only for higher concentrations (0.03 M). The same is true with malate and citrate. When medium concentrations (0.003 M) are used, succinate, fumarate, malate, citrate, and glutamate are all active. At still lower concentrations (0.0003 M) they are all active, but glutamate is only slightly active. Aspartate showed no action. The amount of butyrate which disappears may be 3 or 4 times greater than the amount of added succinate, fumarate, malate, or citrate. This fact is a clear indication that these substances act catalytically. In the experiment shown in Table I α glycerophosphate was added because it was found that in some preparations it accelerated the disappearance of butyrate.

Formation of Phosphopyruvate—It was previously found (1) that phosphopyruvate or a similar substance accumulated when fumarate was added, but not when fatty acids were also present. The formation of phospho

pyruvate from substances which are active in promoting butyrate oxidation was therefore studied. As is shown in Table II, phosphopyruvate was formed from succinate, fumarate, malate, citrate, and also in smaller amounts from glutamate. In the presence of fluoride (0.02 M) the formation is practically the same in all cases, the only difference being that there occurs a greater disappearance of inorganic phosphate. There is also a greater uptake of inorganic phosphate with all the additions as compared with the control.

TABLE II
Formation of Phosphopyruvate

Composition of the system: 2.5 ml of liver enzymes + 0.2 ml of M/15 phosphate buffer of pH 7.7 + 0.1 ml of 0.1 M magnesium chloride + 0.5 mg of cytochrome c preparation + 1 mg of adenylic acid + 0.2 ml of 0.1 M additions. Total volume, 6 ml. 90 minutes at 25° in oxygen. The results are given in micromoles.

Additions	Inorganic P	P liberated by		Pyruvic acid	
		HgCl ₂	Hypoiodite	Before	After 60 min at 100 in N HCl
None	18.3	0	0.4	0	1.5
" + 0.02 M NaF	8.6	0.7	0.1	0	1.9
Succinate	12.6	3.7	3.2	0	5.3
" + 0.02 M NaF	2.9	4.9	4.0	1.6	8.5
Fumarate	11.1	4.9	4.2	0	6.8
" + 0.02 M NaF	2.3	5.5	4.8	1.0	8.4
Malate	12.1	3.5	3.7	0	5.3
" + 0.02 M NaF	2.9	4.9	4.2	0.6	7.2
Citrate	8.2	5.8	5.3	0	8.2
" + 0.02 M NaF	3.3	4.2	3.3	2.6	8.0
Glutamate	14.4	1.5	1.4	0	2.5
" + 0.02 M NaF	5.1	1.9	1.1	0	3.6

The estimation of phosphopyruvate by the hypoiodite and mercuric chloride methods gave practically the same results. The amount of pyruvate liberated by acid hydrolysis gave higher values, presumably because of some interfering substance, but the results were parallel with those obtained by other methods. No pyruvate was present before hydrolysis except in the presence of fluoride plus succinate, fumarate, malate, and citrate, when a small amount accumulated. Smaller amounts of succinate and fumarate, while still promoting the oxidation of butyrate, do not lead to the accumulation of detectable amounts of phosphopyruvate.

The addition of 3-phosphoglycerate produced a formation of phosphopyruvate which was completely suppressed by 0.02 M fluoride. This shows

that enolase is inhibited by that concentration of fluoride and therefore that phosphopyruvate may be formed by a mechanism in which enolase is not involved. No phosphopyruvate was formed from lactate or pyruvate.

Components Necessary for Formation of Phosphopyruvate—As is shown in Table III, no phosphopyruvate is formed from fumarate in the absence of cytochrome c, adenylic acid, or inorganic phosphate. Adenylic acid is also necessary for phosphopyruvate formation from succinate or citrate.

Action of Malonate on Phosphopyruvate Formation—As malonate is known to inhibit succinic dehydrogenase, it was considered that it might be useful as a means of finding out which substance is the immediate precursor of phosphopyruvate. If it were fumarate, an inhibition of phosphopyruvate formation from succinate but not from fumarate would be expected. However, it was found (Table IV) that malonate inhibits phosphopyruvate formation from succinate, fumarate, and citrate approximately to the same

TABLE III

Components Necessary for Formation of Phosphopyruvate

The complete system was as in Table II with fumarate. The results are given in micromoles

	Inorganic P	P liberated by hypodite	Pyruvic acid liberated by acid
Complete system	9.3	5.8	6.5
No cytochrome	18.9	0.3	0.4
" adenylic acid	16.1	0.9	0.6
" phosphate	3.4	1.0	1.6
" fumarate	19.0	0.6	0.5

degree. At 0.001 M there is almost no inhibition and with a higher concentration (0.005 M) formation of phosphopyruvate is almost completely inhibited.

Influence of Phosphopyruvate on Butyrate Disappearance—As phosphopyruvate is formed from the compounds which promote butyrate oxidation, the action of synthetic phosphopyruvate was studied. Curiously enough it was found that phosphopyruvate is active only in a medium containing bicarbonate (Tables V and VI). If phosphate buffer was used and the carbon dioxide was absorbed with alkali, hardly any disappearance of butyrate occurred. This effect is not due to differences in the pH of the medium, as was proved by measurements with a glass electrode. Moreover, the bicarbonate buffer did not affect the butyrate oxidation when fumarate instead of phosphopyruvate was used.

The rate of butyrate and phosphopyruvate disappearance with and without bicarbonate was studied (Table V). Phosphopyruvate disappeared

within 20 minutes in the presence of bicarbonate and butyrate. However, butyrate continued to disappear even after phosphopyruvate was not detectable any more. In the absence of bicarbonate the disappearance of phosphopyruvate was slower and hardly any butyrate was oxidized.

It was found that the disappearance of butyrate in the presence of phosphopyruvate occurred only with cytochrome *c*, adenylic acid, and bicarbonate present (Table VI). Without added inorganic phosphate there was

TABLE IV

Action of Malonate on Phosphopyruvate Formation

The complete system was as in Table II. The results are given in micromoles of phosphate liberated by hypiodite.

	Malonate concentration			
	0	0.001 M	0.003 M	0.005 M
Succinate	2.8	2.6	1.3	0.1
Fumarate	2.0	2.0	1.2	0.8
Citrate	6.3	4.7	2.2	1.1

TABLE V

Influence of Carbon Dioxide on Butyrate and Phosphopyruvate Disappearance

Composition of the system: 2.5 ml of liver enzymes + 0.2 ml of M/15 phosphate buffer of pH 7.7 + 0.1 ml of 0.1 M magnesium chloride + 0.5 mg of cytochrome *c* preparation + 1 mg of adenylic acid + sodium phosphopyruvate + 16.9 micromoles of butyrate. Series A, + 0.3 ml of 0.15 M sodium bicarbonate, gas, 2 per cent carbon dioxide in oxygen. Series B, no bicarbonate, oxygen, CO₂ absorbed with alkali. Total volume, 6 ml. The results are given in micromoles.

		Time					
		0 min	10 min	20 min	30 min	60 min	90 min
Series A (with CO ₂)	Butyrate	16.9	16.8	15.4	14.5	11.7	9.5
	Phosphopyruvate	1.8	0.3	0	0	0	0
Series B (no CO ₂)	Butyrate	16.9	16.7	16.6	15.6	14.7	15.0
	Phosphopyruvate	2	1.2	1.4	1.0	0.6	0.1

a small disappearance, but the enzyme preparation already contained a certain amount of phosphate. In the experiment shown in Table VI the estimations of phosphopyruvate were carried out after 40 minutes of incubation, whereas butyrate was estimated after 90 minutes, as the former compound disappears faster than the latter.

Phosphopyruvate disappears faster when butyrate is oxidized. The absence of any of the components necessary for the oxidation slows down the

rate of disappearance. In the absence of bicarbonate, phosphopyruvate generally produces an inhibition of the small butyrate oxidation which occurs when no phosphopyruvate is added.

Influence of Pyruvate and Lactate—Both these substances increase the rate of butyrate oxidation. However, in the majority of experiments they were less active than fumarate or phosphopyruvate plus carbon dioxide. Their action, in contrast to that of phosphopyruvate, is not influenced by the presence of carbon dioxide. In some experiments the disappearance

TABLE VI

Disappearance of Butyrate and Phosphopyruvate

Composition of the complete system as in Table V, Series A. Time of incubation at 25°, 40 minutes for phosphate estimation, 90 minutes for butyrate. The results are given in micromoles.

	Butyrate disappearance	Inorganic P	Phosphopyruvate
Complete system (initial)		15.1	5.5
" "	9.1	20.6	1.8
No cytochrome	0	20.2	3.3
" phosphate	6.7	9.8	0.5
" adenylic acid	0	19.1	2.4
" phosphopyruvate	1.2	17.5	0.4
" bicarbonate	0	17.8	4.6
" butyrate		19.5	3.5

TABLE VII

Oxygen Uptake and Formation of Acetone Bodies

Complete system as in Table II, 2 micromoles of fumarate, 90 minutes at 25°. The results are given in micromoles.

	Oxygen uptake	CO ₂ formed	Δ butyrate	Acetoacetate	β Hydroxy butyrate
Complete system, no butyrate	2.4	2.2		0	0.8
Same + 18 micromoles butyrate	43.4	23.2	9.6	6.8 7.2*	5.8

* Aniline method

produced by addition of lactate or pyruvate was increased by bicarbonate, but the blank was affected to the same extent.

Oxygen Uptake and Reaction Product—In a previous paper (1) the oxygen uptake was measured in the system with a high concentration of fumarate. Under these conditions the oxygen uptake of the blank with no butyrate was rather high. After subtraction of this blank value, it was found that about 1 molecule of oxygen per molecule of butyrate was used up. The amount of acetoacetate formed was small.

In the later experiments in which a low concentration of fumarate was used, the oxygen uptake of the blank was much lower. This gives more significance to the results, as it is doubtful whether the oxygen uptake of the blank should be subtracted from the uptake with butyrate.

The results of an experiment in which both oxygen uptake and the ketone bodies formed were determined are reproduced in Table VII. The amount of acetoacetate formed per molecule of butyrate was 0.7 molecule, and there was good agreement between the aniline and the modified Van Slyke methods. About 0.5 molecule of β -hydroxybutyrate was formed. Therefore it can be considered that all the butyrate which disappears is transformed into ketone bodies. The oxidation of butyrate to acetoacetate should require 1 molecule of oxygen. The uptake of oxygen actually found was about 4 molecules per molecule of butyrate. These high values, ranging from 3 to 4, have been observed in many experiments. The carbon dioxide formed was about 2.2 molecules per molecule of butyrate.

The identity of the substance which is oxidized together with butyrate has not been established.

DISCUSSION

The rate of butyrate oxidation is increased by substances which intervene in the Krebs citric acid cycle. The same substances give rise to phosphopyruvate. The latter substance is also active, but only in the presence of carbon dioxide. It is difficult to ascertain which is the active substance, because enzymes are present which catalyze their interconversion. For phosphopyruvate it appears probable that there might occur a carboxylation to a phosphorylated C_4 compound. A carbon dioxide fixation on a phosphorylated compound has been suggested by Werkman and Wood (10). As to the type of reaction between the active compound and butyrate nothing is known. Butyrate appears to be oxidized to acetoacetate without an intermediary formation of β -hydroxybutyrate. Most preparations formed acetoacetate at a higher rate from butyrate than from β -hydroxybutyrate and in some enzyme preparations β -hydroxybutyrate was not oxidized, while butyrate was. Similar results were obtained by Jowett and Quastel (11) in liver slices.

The high oxygen uptake and carbon dioxide formation show that some other substance is oxidized together with butyrate. Either there is a coupled reaction or butyrate acts by maintaining the activity of some part of the system necessary for the oxidation of other substrates.

SUMMARY

Succinate, fumarate, malate, citrate, and glutamate increase the rate of butyrate oxidation by preparations of liver enzyme. These same sub-

stances give rise to a formation of phosphopyruvate. The latter was also active, but only in the presence of carbon dioxide, whereas pyruvate and lactate were less active. No phosphopyruvate was formed in the absence of adenylic acid, cytochrome *c*, or inorganic phosphate. Malonate at different concentrations equally inhibited phosphopyruvate formation from succinate, fumarate, or citrate.

Butyrate was nearly all recovered as acetoacetate, but the amount of oxygen used was greatly in excess for this reaction.

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PYRIMIDINE NUCLEOSIDES AND NUCLEOTIDES AS GROWTH FACTORS FOR MUTANT STRAINS OF *NEUROSPORA**

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By means of x-ray and ultraviolet treatment Beadle and Tatum have produced mutant strains of *Neurospora* which differ from the normal wild type in their inability to grow on a simple medium containing salts, sugar, and biotin (1) In many instances it has been found that the medium may be made adequate for normal growth by the addition of yeast extract or a specific, well known cell constituent such as a water-soluble vitamin or an essential amino acid It appears, therefore, that the new mutant is unable to synthesize the missing constituent and from the genetic evidence in the cases examined is differentiated from the normal by a single gene

Among the new mutants isolated was one, Strain 1298, that in preliminary experiments by Tatum and Beadle appeared to require uracil as a growth factor (2) Further experiments with this strain and with another pyrimidine-deficient mutant, Strain H263,¹ by the present authors showed that uracil was an effective growth factor, and, in fact, superior to yeast extract A systematic examination of the constituents of yeast nucleic acid as growth factors for these mutants showed that the pyrimidine nucleosides or nucleotides were from 10 to 60 times as active as uracil Cytosine and thymine were completely ineffective for one strain, No 1298, but provided for some growth on the other, Strain H263 Other pyrimidines or related compounds examined for growth activity included 5-aminouracil, isocytosine, orotic acid (uracil-4-carboxylic acid), barbituric acid, methylcyanacetylurea, a mixture of the isomeric 2,6-aminochloropyrimidines, 2-methoxy-6-aminopyrimidine, thiamine hydrochloride, adenine, guanine, and xanthine Of these substances, only orotic acid proved to have appreciable activity, the others either failed entirely to promote growth or the amount of growth was negligible Both mutants produced conidia as well as mycelial growth on all active supplements

EXPERIMENTAL

Measurement of Growth—The composition of the basal medium has been described in detail by Horowitz and Beadle (3) The stock cultures were

* Aided by a grant to Professor G W Beadle from the Nutrition Foundation, Inc

¹ This mutant, Strain H263, was isolated by F P Hungate and kindly made available for this work

maintained on agar slants containing the basal medium supplemented with 5 mg of uracil per 10 ml. In most of the quantitative experiments growth was measured by the rate of progression of the mycelia on agar medium in horizontal tubes at 25° (1). These were prepared by adding the supplement in each case to the basal medium containing 3 per cent of pyridine-extracted agar. Inoculations were made by means of a sterile loop from a spore suspension in sterile distilled water. The tubes were marked for the first time about 24 hours after inoculation to allow sufficient time for the mycelial front to become well established. After this they were marked either at 12 or 24 hour intervals over a period of 3 or 4 days, and the average rate in mm per hour was calculated. As rates of less than 1 mm per hour could not be measured with any degree of accuracy over the usual 12 hour period, they were calculated from the total distance covered by the mycelia during the experiment.

Materials—The samples of the pyrimidine nucleotides were obtained from yeast nucleic acid after hydrolysis with ribonuclease as described by Loring and Carpenter (4) and also after alkaline hydrolysis ((5) p 218). Cytidylic acid was used in the form of the free acid, uridylic acid as the crystalline diammonium salt.

Cytidine was prepared from pure cytidylic acid after acid hydrolysis, essentially as given by Levene (6). 1.5 gm of cytidylic acid dissolved in 3 ml of 10 per cent (by weight) sulfuric acid in a sealed tube were hydrolyzed by heating in an oil bath at 130° for 4 hours. The hydrolysate was freed of phosphate and sulfate by means of barium hydroxide, and cytidine was precipitated as the picrate. The latter after recrystallization was converted to the sulfate by the usual procedure ((5) p 165). 265 mg of a product which melted with decomposition at 222° were obtained. After two recrystallizations the decomposition point was raised to 232–233° when the sample was placed in the bath at 228°.

The conditions of hydrolysis mentioned for the preparation of cytidine are those given by Levene and coworkers ((7), (5) p 202) for the conversion of cytidylic acid to cytosine. However, in the publication cited above (6) cytidine was isolated under these conditions. In an experiment in which 100 gm of yeast nucleic acid were autoclaved with 10 per cent sulfuric acid in an attempt to prepare cytosine, only 20 mg of the free base were obtained. When the mother liquors were worked up for cytidine sulfate, 320 mg were isolated.

Cytidine sulfate was also prepared by the method of Brederick *et al* (8). The yield from 100 gm of nucleic acid was 1.1 gm.

Uridine was not isolated but was prepared in solution by the treatment of cytidine sulfate with nitrous acid ((5) p 169). Excess nitrous acid was removed by evaporation to dryness *in vacuo*. A quantitative conversion to uridine was assumed.

Uracil was in part obtained from a commercial source (Eastman Kodak Company) and in part synthesized by the method of Davidson and Baudisch (9). The product obtained by the latter procedure after recrystallization melted with decomposition at 336° .

Cytosine, the isomeric 2,6-aminochloropyrimidines, and 2-methoxy-6-aminopyrimidine were synthesized by the method of Hilbert and Johnson (10). From an initial 25 gm of uracil 2.15 gm of free cytosine with a decomposition point of 318° were obtained. It was also prepared by the hydrolysis of cytidine sulfate as given by Hunter and Hlynka (11) for the preparation of cytosine from yeast nucleic acid. 500 mg of cytidine sulfate dissolved in 5 ml of 20 per cent hydrochloric acid were heated in a sealed tube at 175° for 2 hours. The excess hydrochloric acid was removed *in vacuo*, and the residue was redissolved in water and treated with an excess of hot barium hydroxide. The suspension was distilled *in vacuo* to remove ammonia, and the barium was removed quantitatively with sulfuric acid. The filtrate was decolorized with norit and concentrated to a small volume *in vacuo*. 64 mg of uracil, which melted with decomposition at 332° , crystallized from the solution. The filtrate was further concentrated, and a small amount of insoluble material filtered off. When the latter filtrate was made alkaline with concentrated ammonium hydroxide, a typical crystalline precipitate of cytosine separated. After recrystallization from dilute ammonia 52 mg of cytosine with a decomposition point of 315° were obtained. A mixture with the synthetic product showed no depression of the decomposition point.

Thymine and methylcyanacetylurea were synthesized by the method of Bergmann and Johnson (12). The melting points of the two latter compounds were $318-319^{\circ}$ and 192° respectively. Samples of synthetic cytosine, isocytosine, and orotic acid were kindly provided by Professor T. B. Johnson.²

Results of Growth Experiments—The kind of growth response found varied with the nature of the supplement. With the more active factors, constant growth rates at different levels for the different concentrations were reached 12 or 24 hours following the initial lag period. When uracil or orotic acid was used, however, a phenomenon of adaptation was observed (13). Whereas the initial rate on a suboptimal concentration was low or even negligible, the values increased progressively with time. The behavior of mutant Strain 1298 on different levels of uridylic acid and uracil is shown graphically in Fig. 1. For comparison, a curve showing the behavior of the normal or wild type *Neurospora* on the basal medium supplemented with 10 mg of uracil per 10 ml (Curve a) is also shown. The average growth rates on different uracil and uridylic acid concentrations for each 12 hour incubation period were plotted against time as the mycelial

² We should like to express our thanks to Professor Johnson for these samples.

front advanced down the length of the tube. With both uracil and uridylic acid maximum growth rates comparable to that of the wild type were obtained at levels of 10 and 1 mg respectively per 10 ml of medium (Curves *b* and *c*). With 0.08 mg of uridylic acid per 10 ml, a constant growth rate was established at about 1.4 mm per hour (Curve *e*). With less than the optimal concentration of uracil, however, i.e. 2 or 3 mg per 10 ml (Curves *d* and *h*), the rate increased with time, and the maximum was obtained after about 72 hours. With a concentration of 1 mg of uracil per 10 ml, no signs of growth were evident for about 48 hours (Curve *f*), but after this a progressively increasing rate of growth was observed. It was

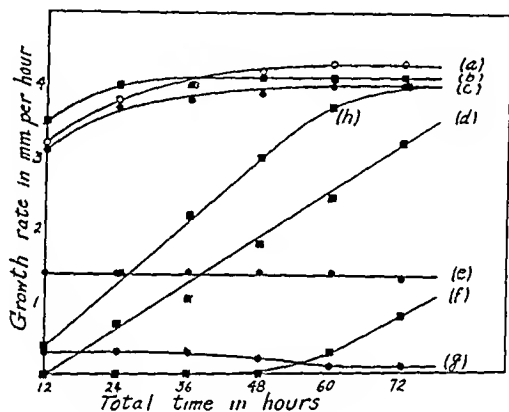


FIG 1 Rates of growth of the mutant Strain 1298 on uracil and uridylic acid after different incubation periods. Concentrations are expressed as mg per 10 ml of basal medium. Curves *b*, *h*, *d*, and *f*, 10, 3, 2, and 1 mg of uracil respectively, Curves *c*, *e*, and *g*, 1, 0.08, and 0.025 mg of uridylic acid respectively, Curve *a*, wild type control on 10 mg of uracil.

not determined, however, whether or not, if given sufficient time, the maximum rate would have been obtained in this case. As the concentrations are increased 2- or 3-fold above the optimum values, maximum growth rates are also found. When the concentration of uracil was increased to 100 mg per 10 ml, however, a definite inhibition of growth was observed.

The maximum rates of growth of the two mutants on each of the different supplements are summarized in Table I. Several striking differences between the two mutants are apparent from these results. While Strain 1298 fails completely to grow on the basal medium, Strain H263 grows at a rate of approximately 0.25 mm per hour. The latter strain, in contrast to Strain 1298, also shows an appreciable ability to utilize cytosine, thy-

mine, and yeast nucleic acid Unlike Strain 1298, however, it has failed to show as high a maximum rate of growth as that found for the wild type

The relative efficiency of uridine, cytidine, uridylic acid, cytidylic acid, and uracil in promoting growth of the mutant Strain 1298 is shown in Fig 2 The growth rates were plotted as a function of the logarithm of the concentration expressed as micrograms per 10 ml of medium The points shown are the averages of two or more experiments in each case From these results it is apparent that uridine is the most active growth factor for this mutant, promoting maximum growth at a level of 160 γ per 10 ml Cytidine is slightly less active, maximum growth being obtained at about

TABLE I

Maximum Rates of Growth of Mutant Strains 1298 and H263 on Various Supplements

Supplement	Concentration in medium	Rate of growth	
		Strain 1298	Strain H263
	<i>mg per 10 ml</i>	<i>mm per hr</i>	<i>mm per hr</i>
Uracil	10	4 0, 4 0, 4 1*	2 8, 3 2, 3 3
Cytosine	10	0	1 2, 1 4
Uracil + ribose†	0 35 uracil, 0 46 d-ribose	0	
Cytosine + ribose	10 cytosine, 10 d ribose	0	1 2
Thymine	10	0	1 2, 0 9, 1 4
Orotic acid	7	4 1	3 3
Cytidine sulfate	0 9	4 2, 4 2	3 6, 3 3
Uridine	0 75	4 2, 4 3	
Cytidylic acid	1 0	4 1, 4 0, 4 0	3 3, 3 1
Diammonium uridylate	1 2	4 1, 4 0	3 0, 3 0
Yeast ribonucleic acid	2 5	0	3 2, 2 9
Blank		0	0 25

* Each value represents the maximum rate found in a different experiment

† Obtained from the A D Mackay Company

400 γ Uridylic acid allows maximum growth at slightly less than 1 mg per 10 ml, whereas with cytidylic acid 1 mg is required In the case of uracil the points shown represent the maximum rates reached at the different concentrations in experiments of 3 or 4 days duration, under these conditions owing to the adaptation phenomenon mentioned above the maximum rate is obtained with about 5 mg per 10 ml

The results plotted in Fig 2 also show a significant difference in the maximum growth rates for the nucleosides as compared to the nucleotides Here as well uridine is superior to cytidine, whereas uridylic and cytidylic acids give the same value

The inability of mutant Strain 1298 to utilize cytosine for growth as compared to its ability to use cytidine or cytidylic acid and the relatively low activity of uracil suggested that the available *d*-ribose concentration might be a limiting factor in the utilization of the free pyrimidines. Experiments were, therefore, carried out in which *d*-ribose as well as cytosine and uracil were used as supplements. The addition of *d*-ribose in amounts up to 10 mg per 10 ml of medium failed to affect the growth-promoting properties of either uracil or cytosine.

The various other compounds tested, in the case of Strain 1298, included isocytosine, barbituric acid, thiamine hydrochloride, adenine sulfate, guanine, and xanthine. In addition to these, methylecyanacetylurea, isomeric 2,6-aminochloropyrimidines, and 2-methoxy-6-aminopyrimidine

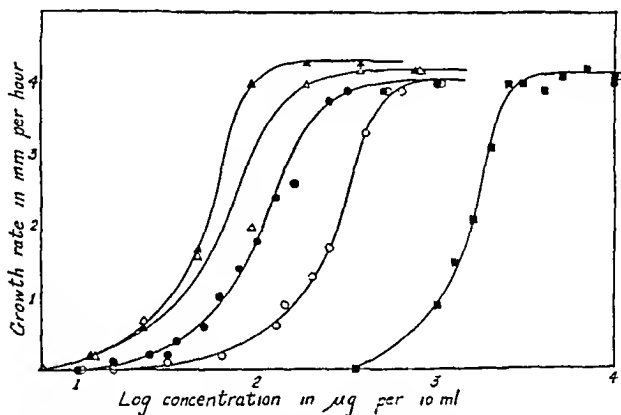


FIG. 2 Rates of growth of the mutant Strain 1298 as a function of concentration: ▲, uridine, △, cytidine, ●, uridylic acid, ○, cytidylic acid, ■, uracil.

were tested on mutant Strain H263. The concentration used in each case was 10 mg per 10 ml of medium. With Strain 1298, as in the blank, no growth was obtained. With Strain H263 rates of growth comparable to that of the blank occurred.

Genetic Analysis—In order to determine whether or not single genes were responsible for the inability of these mutants to synthesize the pyrimidine nucleosides and nucleotides, they were crossed with the normal wild type. The eight ascospores from each of twenty asci of the Strain 1298 cross and from each of nine of the Strain H263 cross were isolated in order, germinated on medium supplemented with cytidylic acid, and their identity determined by transferring to unsupplemented basal medium. Of each set of eight ascospores examined, four failed to grow in the absence

of the pyrimidine supplement, while the other four grew like the normal wild type. These results indicate that both mutants differed from the normal in synthetic ability by one gene³

Heterocaryon Formation—The different growth response of the two mutants to the various supplements mentioned above indicated a difference in their genetic constitution. In other such cases in which two *Neurospora* mutants are unable to synthesize an essential constituent, it has been shown that the formation of a heterocaryon between the two (*i.e.* hyphae in which two or more genetic types are present) produces a new strain, which now is able to grow on the basal medium alone (2). In such instances, it appears that the reaction by which the missing constituent is synthesized is blocked at different steps in the two strains. A combination of the two as a heterocaryon allows the complete synthesis to be effected, and the new strain behaves like the wild type in its ability to grow without added supplement.

When small portions of mycelium from Strains 1298 and H263 of the same mating type were placed together on the basal medium, it was found that growth comparable to that of the wild type took place.⁴

DISCUSSION

Although free pyrimidines have been reported previously as growth factors for several different microorganisms (14-16), it does not appear that the availability of the corresponding naturally occurring nucleosides and nucleotides has been studied. From the results presented above it seems possible that the latter will prove more active in those cases, as they have for the pyrimidine-deficient *Neurospora* mutants. It is reasonable to assume that such microorganisms are unable to synthesize sufficient nucleic acid for normal cell development and growth owing to their inability to synthesize the pyrimidine components.

The relatively low availability of the free pyrimidines for the growth of these *Neurospora* mutants compared to the nucleosides and nucleotides is in agreement with the poor utilization of free pyrimidines which has been reported in higher animals (17). As nucleic acid and the pyrimidine nucleosides are readily metabolized under the latter conditions (17-18), the

³ These experiments on Strains 1298 and H263 were performed by G. W. Beadle and F. P. Hungate respectively.

⁴ The growth requirements of a third mutant, Strain 45203, supplied to us by Professor Beadle were also investigated. It was found to grow on hydrolyzed yeast nucleic acid, on uracil, cytidine sulfate, cytidylic acid, and uridylic acid at rates similar to those obtained for Strain 1298. As the new mutant failed to grow on cytosine and thymine and did not form a heterocaryon with Strain 1298, it appeared identical with the latter and therefore provides an example of a reoccurrence of the same gene mutation.

results of both types of experiments show a greater importance for the nucleosides and nucleotides in the metabolism of nucleic acid. This is particularly striking in the case of mutant Strain 1298, which although completely unable to utilize cytosine, nevertheless grows well on cytidine or cytidylic acid. Here, although this mutant is unable to deaminate cytosine to uracil, it is evidently able to convert cytidine to uridine or vice versa. In this instance and probably with other strains of *Neurospora* as well, it appears unlikely that free cytosine functions as an intermediate in the synthesis of cytidine and cytidylic acid.

The use of choline-deficient, pyridoxine-deficient, and *p*-aminobenzoic acid-deficient mutants in methods of assay for choline, pyridoxine, and *p*-aminobenzoic acid, respectively, has been reported (3, 19, 20). In a similar manner it is possible to use the mutants described in the present paper to determine total pyrimidine nucleoside and nucleotide concentration in terms of any one of the more active compounds. The determination of the individual nucleosides or nucleotides, however, must await the development of a satisfactory fractionation procedure.

SUMMARY

The growth requirements of two experimentally produced mutant strains of *Neurospora*, Nos. 1298 and H263, which are unable to grow on a basal medium containing simple salts, sugar, and biotin have been studied. It has been shown that when the pyrimidine compounds which occur in yeast ribonucleic acid are used as supplements, normal mycelial growth and production of conidia result. The nucleosides, uridine and cytidine, and the nucleotides, uridylic acid and cytidylic acid, are from 10 to 60 times as active as the free pyrimidines, uracil or orotic acid. Cytosine and thymine are completely inactive for one mutant, Strain 1298, but provide for some growth for the other, Strain H263.

The difference in the availability of the free pyrimidines for the growth of the two mutants, the production of a heterocaryon when they are allowed to fuse, and the types of segregations obtained after crosses with the normal wild type are consistent with the theory that pyrimidine synthesis in these microorganisms involves several reactions, each of which is controlled by a single gene.

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A METHOD FOR THE DETERMINATION OF GLUTAMIC ACID IN PROTEINS

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This method is based on the fact that glutamic acid can be converted to pyrrolidonecarboxylic acid under conditions which do not affect other amino acids, except cystine and cysteine. The corresponding decrease in amino nitrogen, corrected for cystine, can be used to estimate the amount of glutamic acid in protein hydrolysates. By the use of the Van Slyke manometric apparatus, the method is applicable to the determination of glutamic acid in 25 to 100 mg. samples of protein with an estimated accuracy of 5 per cent.

Several attempts to use the glutamic acid-pyrrolidonecarboxylic acid reaction for a quantitative estimation of glutamic acid and glutamine have been recorded. Vickery (1) suggested boiling protein hydrolysates at pH 3 for a sufficient length of time to convert glutamic acid to pyrrolidonecarboxylic acid, extracting the latter with ethyl acetate, reforming glutamic acid from it by acid hydrolysis, and isolating the glutamic acid hydrochloride. Wilson and Cannan (2) described the behavior of the glutamic acid-pyrrolidonecarboxylic acid system in detail and recommended that solutions be adjusted to pH 4 and heated for 50 hours at 100° or 6 hours at 120° in order to obtain optimal rate and extent of transformation of glutamic acid to pyrrolidonecarboxylic acid. In attempting to use this method for the determination of glutamic acid they found that protein hydrolysates, when boiled for several days at 100°, lost far more amino nitrogen than could be accounted for by the amount of glutamic acid present, a fact which they attributed to anhydride formation. Pucher and Vickery (3) determined glutamine by converting it to pyrrolidonecarboxylic acid with heat in neutral solution, extracting the latter with ethyl acetate at pH 2.4, and determining the amino nitrogen in the extract before and after hydrolysis with hydrochloric acid. The increase was a direct measure of the glutamine originally present. This method did not yield satisfactory results for Neuburger and Sanger (4) who, instead, determined glutamine by measuring the carbon dioxide developed by ninhydrin treatment both before and after heating for 2 to 3 hours at pH 6.8.

Isolation procedures have been used in the determination of most of

the recorded values for the glutamic acid content of proteins Chibnall and his coworkers (5) have refined the Foreman procedure (6) to a claimed accuracy of 1 or 2 per cent but the method is lengthy and laborious Glutamic acid has also been determined by the isotope dilution technique (7) and by an enzymatic method (8) in which glutamic acid is converted to succinic acid by oxidation and the latter is determined in the Warburg apparatus A microbiological method has recently been suggested (9) The present procedure appears to have the advantages of rapidity and convenience over those previously described

EXPERIMENTAL

Effect of pH and Time of Autoclaving on Amino Nitrogen Loss of Glutamic Acid—The following experiments at 125° gave results similar to those observed by Wilson and Cannan (2) at 100° and 120° A series of glutamic acid solutions was made up at final reactions ranging from pH 2 to 6 by the addition of acid or alkali The 0.5 per cent glutamic acid solution had a pH of 3.3 (All pH determinations were made by the use of the glass electrode) Residual amino nitrogen was determined by the Van Slyke manometric method after 1, 2, 3, and 4 hours of autoclaving at 125° The results indicated that within the pH range of 2.5 to 4, 92 to 96 per cent of the glutamic acid was changed to pyrrolidonecarboxylic acid after 4 hours The reaction pH 3.3 was chosen as the preferred hydrogen ion concentration as a compromise between rapidity (pH 3.0) and completeness (pH 4.0) of conversion (2) A larger series was run with glutamic acid (pH 3.3) for periods of time up to 15 hours As shown in Table I, equilibrium was approached in 4 hours In designing a method, it did not appear to us to be necessary to reach an exact equilibrium, whenever the rate of change of glutamic acid to pyrrolidonecarboxylic acid had become so slow that the percentage of residual amino nitrogen was reproducible, the analysis could be made reasonably quantitative In practice, a period of 4 hours in the autoclave was adopted

When glutamic acid solutions were autoclaved, a decline in pH of approximately 1 unit occurred However, in the assays of proteins, the pH remained within 0.5 unit of the adjusted value, the unaffected amino acids acting as buffers It is appreciated that the conditions of pH, time, and temperature of autoclaving are not necessarily optimal for this reaction, they have, however, been convenient in our hands

Effect of Autoclaving on Individual Amino Acids—The amino acids used were commercial preparations except for glutamic acid, aspartic acid, and cystine, each of which had been recrystallized five times Solutions of the amino acids or their hydrochlorides were made up to contain 5 mg per ml When necessary, acid was added to facilitate solution 5 ml aliquots were

titrated with 0.1 N acid or alkali to determine the amount necessary to bring the pH to 3.3. A second aliquot was pipetted into a 25 ml volumetric flask, and the acid or alkali added as found necessary. The mixture was then diluted to about 15 ml, autoclaved for 4 hours at 125–126°, cooled, and diluted to volume. No detectable loss (less than 1 per cent) of amino nitrogen occurred with tryptophane, lysine, histidine, arginine, tyrosine, methionine, serine, threonine, glycine, alanine, and aspartic acid. No amino nitrogen developed in solutions of proline or hydroxyproline. Cystine lost approximately 50 per cent and cysteine 20 per cent of their original "amino nitrogen." These anomalies will be discussed in greater detail below.

Six separate determinations (each in duplicate) of glutamic acid showed the following percentage losses: 91.8, 91.0, 92.4, 92.4, 91.5, 92.3, average 91.9. In the calculations which follow it has been assumed that the decrease of

TABLE I
Loss of Amino Nitrogen in Glutamic Acid Solutions Autoclaved at 125°

Time	Residual amino nitrogen	Amino nitrogen loss
hrs	mg per ml	per cent
0	0.193	
1	0.087	55
2	0.037	80
3	0.023	88
4	0.016	92
7	0.013	93
15	0.013	93

amino nitrogen due to glutamic acid represents 92 per cent of that originally present.¹

Recovery of Glutamic Acid from Mixtures of Amino Acids (Exclusive of Cystine and Cysteine)—The experiments outlined in Table II indicated that the cyclization of glutamic acid was not appreciably affected by the presence of mixtures of amino acids (exclusive of cystine and cysteine) similar to those obtained by protein hydrolysis. The slight decrease in the amino nitrogen content of the amino acid mixtures without glutamic acid is possibly ascribable to traces of glutamic acid, cystine, or other extraneous compounds in the preparations. One run with hydrolyzed gramicidin, known to contain no glutamic acid or cystine (10), indicated that mixtures

¹ The factor to be used for calculation with protein hydrolysates can best be estimated for any given run by determining the loss of amino nitrogen in a solution of glutamic acid containing an equivalent amount of salt and autoclaved simultaneously.

of amino acids, at least as they occur in this substance, do not lose appreciable amounts of amino nitrogen during autoclaving (amino N, before autoclaving, 0.103 mg per ml, after, 0.102 mg per ml)

Magnitude of Cystine Correction—Two samples of cystine, one carefully purified ($[\alpha]_D^{25} = -213^\circ$ in 1.0 N HCl), the other a commercial product, gave similar results. As shown in Table III, approximately 130 per cent of the calculated amino nitrogen was determinable by the manometric Van Slyke procedure. Kendrick and Hanke (11) reported about 140 per cent. After 4 hours at 125° and at pH 3.3, the amino nitrogen loss was equivalent to 50 per cent of the observed value, or 65 per cent of the theoretical value. The loss in amino nitrogen due to cystine in mixtures of amino acids and protein hydrolysates varied from 47 to 70 per cent of theory (Table III). From these data we have chosen arbitrarily to use the value of 65 per cent as an approximation in glutamic acid analyses.

The following assumptions have been made in calculation of the correction due to the behavior of cystine: that there is a loss of approximately 10 per cent of the original cystine of a protein after 48 hours of hydrolysis with 20 per cent hydrochloric acid, as demonstrated with insulin by Miller and du Vigneaud (12), and that the residual cystine is present as such whether arising from cystine or cysteine originally. These assumptions have been shown to be correct for gliadin when determined by a modification of Vassel's method (13).

Method of hydrolysis	Cystine	Cystine + cystine
	<i>per cent</i>	<i>per cent</i>
HCOOH HCl, 24 hrs	0.23	2.13
20% HCl, 24 hrs	Trace	2.15
20% " 48 "	"	1.97

Most proteins contain approximately 10 times as much glutamic acid as cystine, hence errors in the assumptions mentioned above would be within the expected error of the method. For keratins and insulin, the same protein hydrolysate probably should be used for separate glutamic acid and cystine analyses, in order to make the cystine correction more accurate. In calculating the glutamic acid content of proteins (48 hours hydrolysis), we have found it convenient to subtract 0.78 mg. for each 1 mg. of cystine plus cysteine known to be present.*

The nature of the reactions which occur in the cystine solutions at pH 3.3 and 125° has not been studied in detail. One solution contained, after

* Each mg. of cystine would account for a loss of $0.9 \times 14/120 \times 0.65 = 0.068$ mg. of amino nitrogen, which would in turn be calculated as $0.068 \times 147/14 \times 100/92$ or 0.78 mg. of glutamic acid.

TABLE II

Recovery of Glutamic Acid from Mixtures of Amino Acids (Excluding Cystine and Cysteine)

Amino acid mix No	Glutamic acid added mg $\text{NH}_2\text{-N}$ per ml	Amino nitrogen*		Glutamic acid found		
		Original	Autoclaved	$\text{NH}_2\text{-N}$	Difference	Recovery
		mg per ml	mg per ml	mg per ml	mg per ml	per cent
1†	0	0 171	0 166	0 0055		
1	0 095	0 270	0 176	0 102	0 0965	102
2‡	0	0 103	0 1015	0 002		
2	0 0335	0 136	0 104	0 035	0 033	98

* Each figure is the average of four determinations checking within 3 per cent

† Mix 1 contained (in 100 ml) tryptophane 15 mg, tyrosine 40 mg, phenylalanine 20 mg, arginine hydrochloride 50 mg, histidine hydrochloride 20 mg, alanine 50 mg, valine 50 mg, aspartic acid 20 mg, proline 150 mg, hydroxyproline 30 mg, leucine 60 mg, methionine 20 mg, lysine dihydrochloride 20 mg, and threonine 100 mg

‡ Mix 2 contained (in 50 ml) serine 40 mg, threonine 35 mg, leucine 150 mg, isoleucine 150 mg, tyrosine 125 mg, histidine hydrochloride 125 mg, arginine hydrochloride 35 mg, and lysine 15 mg

TABLE III

Loss of Amino Nitrogen from Cystine Solutions Autoclaved 4 Hours at 125°

	Cystine present theoretical mg $\text{NH}_2\text{-N}$ per ml	Amino nitrogen*		Loss due to cystine as per cent of theoretical
		Original	Autoclaved	
		mg $\text{NH}_2\text{-N}$ per ml	mg $\text{NH}_2\text{-N}$ per ml	
Cystine, recrystallized	0 117	0 153	0 077	65
" commercial	0 117	0 1545	0 075	68
" + glycine + glutamic acid†	0 112	0 364	0 184	69
Cystine + casein‡	0 0303	0 1925	0 144	70
" + amino acid Mix 2§	0 0243	0 1325	0 1186	51
" + glutamic acid + amino acid Mix 2	0 0243	0 1675	0 1235	47

* Average of three to five determinations checking within 3 per cent

† Equimolecular quantities, calculated loss for glutamic acid, 92 per cent of 0 112 = 0 103 mg of amino nitrogen per ml

‡ 10 mg of cystine + 90 mg of casein, hydrolyzed 48 hours with 20 per cent HCl
Loss of amino nitrogen from casein hydrolysate, calculated from data in Table IV
Assumed 10 per cent loss of cystine during hydrolysis

§ See foot note to Table II Correction was applied for the loss of the amino nitrogen of the amino acid mixture without cystine

|| Calculated loss for glutamic acid, 92 per cent of 0 0335 = 0 031 mg of amino nitrogen per ml

being autoclaved, only 30 per cent of its nitrogen in the form of cystine, 60 per cent was present as ammonia. The cystine changes were not in equilibrium after 4 hours. A sample of cystine which had been autoclaved for 16 hours contained only 43 per cent of the theoretical amino nitrogen compared with the 65 per cent remaining after 4 hours.

A sample of cysteine hydrochloride was investigated. The standard procedure for amino nitrogen gave 130 per cent of the theoretical value, similar to the result for cystine, but only 20 per cent disappeared during autoclaving.

Possible Interfering Substances (Other Than Amino Acids)—A number of proteins contain small amounts of non-amino acid constituents, the possible interference of which should be emphasized. Among these are glucose, glucosamine, and the products of hydrolysis from nucleic acids, heme, etc. Only the first two of these have been studied briefly. Glucose was added in amounts equivalent to 3 per cent of the protein to a wheat gluten hydrolysate without affecting the loss of amino nitrogen upon autoclaving. On the other hand, a solution of commercial glucosamine lost 20 per cent of its amino nitrogen. Hence, small amounts of carbohydrates probably will not affect glutamic acid determinations by this method, but when glucosamine is present, additional corrections will have to be made.

Procedure—The glutamic acid analyses shown in Tables IV to VI were obtained by the following method. 100 mg samples³ (in duplicate) were weighed into small flanged test-tubes, 2 ml of 6 N HCl were added, and the tubes were heated on the steam bath until solution occurred. They were then equipped with cold finger condensers and immersed to the depth of 1 to 1½ inches in a stirred oil bath kept at 120–125° for 48 hours. After cooling, the contents of the test-tube were transferred to a 10 ml volumetric flask with thorough washing and adjusted to the mark. The solution was then filtered through dry filter paper (Whatman No. 4, 7 cm) to remove the humin, 2 ml of the filtrate were diluted to 10 ml for the control, and a second 2 ml sample was titrated to pH 3.3 with 1 N NaOH, bromophenol blue and a glass electrode being used for the final adjustment, slightly over 2 ml of alkali were required.⁴ A third 2 ml aliquot was adjusted to pH 3.3 with the necessary amount of alkali, as determined above, in a 10 ml volumetric flask, water was added to make a total volume of 7 to 8 ml,⁵

³ By using 5 ml instead of 10 ml volumetric flasks, 25 mg samples would give sufficient material for duplicate amino nitrogen determinations on the control and autoclaved hydrolysates.

⁴ Attempts were made to avoid this titration by adding a citrate buffer (pH 3.3) after approximate neutralization of the acid, however, abnormal losses of amino nitrogen due to the presence of citrate occurred during autoclaving.

⁵ The final volume is not important. Samples of a wheat gluten hydrolysate were diluted 1:5, 1:10, and 1:20 and autoclaved. The loss of amino nitrogen was identical in each case.

and the flask was placed in a steam autoclave for 4 hours at 125–126° (19 to 20 pounds per sq in) measured from the time at which this temperature was attained. After being cooled the solution was diluted to 10 ml. 2 ml aliquots of the control and autoclaved samples were analyzed for amino nitrogen by the standard Van Slyke manometric technique with a 3 minute reaction period. Control and autoclaved samples were analyzed successively in order to minimize variations in the blank. Calibrated pipettes were used. Temperature was recorded to the nearest 0.2°.

The per cent glutamic acid content when 100 mg samples of protein were used was calculated by the formula

$$\% \text{ glutamic acid (dry basis)} = \frac{57,070(N_1 - N_2) - 0.78C(100 - M)}{(100 - M)}$$

in which N_1 equals the original amino nitrogen in mg per ml, N_2 is the amino nitrogen of the autoclaved sample, C is the cystine content of the protein in per cent, and M is the moisture content of the protein sample in per cent.⁶

Most of the proteins were hydrolyzed 48 hours instead of the usual 20 to 24 hours. With casein 24 and 48 hour hydrolysates were compared (Table IV). No detectable differences were noted, in contrast to the observations of Osborne and Guest (14). The assumption that the humin contained insignificant amounts of glutamic acid appeared justified from the experiments of Roxas (15).

The pH of the protein hydrolysates after being autoclaved varied from 2.5 to 4, with most values falling within the range of 2.9 to 3.6. No detectable differences in the analytical values could be traced to variations within this range of the final pH.

Variability of Results with Protein Hydrolysates—A number of determinations were run upon hydrolysates of a commercial casein sample. The results (Table IV) illustrate the variations which occurred in our hands and call attention to an important weakness of the method, namely, that the desired data are differences between analytical results each of which may vary within the limits of expected error for the determination of amino nitrogen. Differences between duplicate pairs of analyses varied as much as differences between separate hydrolysates. It therefore does not seem as important to hydrolyze a number of separate samples in order to approach maximum accuracy as it does to obtain multiple analyses of two or three hydrolysates.

Recovery Experiments with Protein Hydrolysates—The results of experi-

⁶ The constant 57,070 is equal to $50 \times 147/14 \times 100/92 \times 100$, in which 50 represents the dilution factor, 147/14 the conversion from amino nitrogen to glutamic acid, 100/92 the correction for the incomplete conversion to pyrrolidonecarboxylic acid, and 100 the conversion to percentage.

ments in which glutamic acid was added to gelatin and casein are shown in Table V. The recoveries are close enough to 100 per cent to indicate that the method can be used to obtain approximate results for proteins of unknown glutamic acid content.

Glutamic Acid Determination in a Series of Proteins—The data in Table VI were obtained by the method described above. Most of the glutamic acid analyses agree within the range of expected accuracy with those of

TABLE IV

Reproducibility of Amino Nitrogen Determinations in Duplicate and on Duplicate Hydrolysates of Casein before and after Autoclaving 4 Hours at 125°

Hydrolysate No. * and time of hydrolysis		Amino N (single determinations)			Glutamic acid†	Average‡
		Original	Autoclaved	Difference		
	hrs	mg per ml	mg per ml	mg per ml	per cent	per cent
1	48	0.1805	0.1445	0.036	22.8	22.3 ± 0.3
		0.182	0.146	0.036	22.8	
2	48	0.182	0.147	0.035	22.1	
		0.181	0.148	0.033	20.7	
3	48	0.1835	0.147	0.0365	23.0	
		0.180	0.145	0.035	22.1	
4	48	0.182	0.148	0.034	21.4	
		0.184	0.151	0.037	23.3	
5	48	0.181	0.144	0.037	23.3	
		0.1805	0.147	0.0335	21.1	
6	48	0.188	0.145	0.043	27.1	
7	48	0.184	0.146	0.038	24.0	
8	48	0.179	0.145	0.034	22.1	
9	48	0.187	0.149	0.039	24.7	
10	48	0.1825	0.148	0.0345	21.7	23.9 ± 0.9
11	24	0.182	0.1485	0.0335	21.1	
12	24	0.1855	0.146	0.039	24.7	22.1 ± 0.9
		0.1835	0.151	0.0325	20.5	
		0.187	0.152	0.035	22.1	

* Hydrolysates 1 to 5 were hydrolyzed simultaneously, Hydrolysates 6 to 10 were hydrolyzed over separate periods.

† Corrected for cystine content.

‡ Standard error = $\sqrt{\Sigma d^2/n(n-1)}$

other investigators. Because of the uncertainties which have already been mentioned, it is possible that the final figure may be in error by as much as 10 per cent. The true glutamic acid contents can probably be considered as, if anything, less than the recorded data, inasmuch as most errors would tend to increase the apparent glutamic acid content. Included in these errors would be the possible slight loss of amino nitrogen from mixtures of amino acids other than glutamic acid, and the possible presence of protein

constituents other than amino acids which would either themselves lose amino nitrogen or react with amino acids during the autoclaving, with a resultant net loss of amino nitrogen

Chibnall and coworkers (5) have reported analyses for the glutamic acid contents of egg albumin, β -lactoglobulin, and edestin which they considered within 1 or 2 per cent of the true values. In the present analyses, the glutamic acid content of β -lactoglobulin agreed, but that of egg albumin was somewhat higher than was found by these workers, although still within the limit of error of the method. Our sample of edestin appeared to contain less glutamic acid than did Chibnall's

TABLE V
*Recovery of Added Glutamic Acid from Protein Hydrolysates**

Amount of protein used	Glutamic acid added	Total amino N†		Glutamic acid			
		Original	Auto claved	Found‡	Present in protein	Difference	Recovery
mg	mg	mg	mg	mg	mg	mg	per cent
100 Gelatin	0	10.52	9.53	10.3			
90 "	10	10.45	8.76	19.3	9.3	10.0	100
70 "	30	10.21	7.00	36.6	7.2	29.4	98
50 "	50	10.07	5.47	52.4	5.1	47.3	95
30 "	70	9.62	3.45	70.4	3.1	67.3	96
100 Casein	0	9.10	7.35	19.7			
50 "	50	9.20	4.10	58.2	9.8	48.4	97

* The glutamic acid was added prior to hydrolysis

† Average of duplicate hydrolysates

‡ Corrected for cystine on the assumption that the gelatin contained 0.17 per cent, the casein 0.42 per cent (16). These values are so low that variations would fall within the limits of error of the amino nitrogen determinations

The isotope dilution method described by Rittenberg and Foster (7) has the possibility of being very accurate for determining the amino acid contents of proteins. These authors reported 13.0 per cent glutamic acid in fibrin preparation. Through the kindness of Dr. Rittenberg, a sample of this same preparation was made available to us. With correction of the moisture content to that in the sample as used by them, the present method gave 15.2 per cent glutamic acid. This is slightly over the maximal error expected. The origin of the discrepancy is not apparent at present.

The glutamic acid value for tobacco mosaic virus (17.0 per cent) is considerably above that isolated by Ross (5.3 per cent) but of the order of magnitude suggested by his data for amide nitrogen (18). The remote possibility that the products of hydrolysis of the nucleic acid component might interfere has not been investigated.

TABLE VI
Glutamic Acid Content of Proteins and Polypeptides*

Protein	Nitrogen content (Kjeldahl after dry- ing 18 hrs at 105°)	Total cystine content†	No of hydro- lyses‡	Glutamic acid content	
				Found	Previous determinations
	per cent	per cent		per cent	per cent
Egg albumin§		1.8	2	17.0	16.1 (5), 13.3 (17)
Egg albumin	15.0	1.8	2	16.9	
β Lactoglobulin§		3.4	2	21.5	21.5 (5)
Edestin	18.4	1.4	3	18.3	20.7 (5)
Insulin	15.55	12.5	7	19.6	30 (20), 21 (21)
Tobacco mosaic virus	15.7	0.7	2	17.0	5.3 (18)
Fibrin	16.0	1.5	3	16.0	13.7 (5)
Gladin	17.1	2.8	2	45.7	46.9 (5), 45.3 (22), 43 (17)
Zein	15.5	0.9	4	23.5	31.3 (23)
Glutenin	15.7	1.7	2	35.9	25.7 (24)
Cottonseed globulin	14.8	0.7	2	19.6	17.2 (24)
Soy bean glycinin	16.0	0.8	2	21.0	18.5 (17)
Arachin	17.5	1.3	2	24.2	19.5 (17)
Peanut globulin	17.0	1.3	2	24.3	
Casein	14.0	0.4	10	22.0	22.0 (5), 21.8 (8)
Gelatin	18.2	0.2	3	11.7	5.80 (25)
Wool keratin	16.4	11.8	2	16.0	15.3 (26)
Feather keratin	16.1	7.6	2	12.3	9.7 (26)
Gramicidin		0	1	0	0 (10)
Tyrocidine hydrochloride	13.8	0	2	12.5	

* I am indebted to the following who furnished samples of proteins: D. Breese Jones for the samples of dried egg albumin, arachin, and soy bean glycinin used by Jones and Moeller (17) for analyses of glutamic acid by isolation; D. M. Greenberg for edestin; W. M. Stanley for tobacco mosaic virus prepared by differential centrifugation (18); D. Rittenberg for fibrin; E. F. Jansen for gramicidin, tyrocidine, and a solution of crystalline β lactoglobulin; H. P. Lundgren for a solution of crystalline egg albumin; Eli Lilly and Company for crystalline zinc insulin; and the Southern Regional Research Laboratory for cottonseed and peanut globulins.

† The cystine plus cysteine contents of the samples of gladin, glutenin, chicken feather and wool keratins, and peanut and cottonseed globulins were determined by a modification of the Vassel method (13). The other values were taken from the literature, helpfully summarized in most instances by Cohn and Edsall (16).

‡ At least two closely checking pairs of analyses were run on each hydrolysate.

§ Measured volumes of these solutions were pipetted into test tubes and dried in an oven at 95°. From nitrogen analyses, the amount of protein was determined by using 15.76 and 15.58 as the known nitrogen contents of egg albumin and β lactoglobulin, respectively (5). Inasmuch as in these determinations we did not use the 8 to 16 hour digestion period recently recommended by Chibnall, Rees, and Williams (19), as necessary to obtain maximal results for proteins, the glutamic acid contents may be too high by as much as 3 per cent.

|| According to Hotchkiss (10), the molecular weight of tyrocidine hydrochloride is probably some multiple of 1267. If each fragment of this size contained one glutamic acid residue, 11.6 per cent would be present.

The glutamic acid content of insulin (19.6 per cent) was so low compared to the 30 per cent content recently ascribed to it (20, 27) that additional investigation appeared necessary. Reasonable checks were obtained in duplicate analyses of seven separate hydrolysates. In order to determine whether the high cystine content might have interfered, the loss of amino nitrogen from a mixture of amino acids similar in composition to that of insulin was determined (see Tables II and III, amino acid Mix 2) with and without the correct proportions of added glutamic acid and cystine. In this case cystine appeared to lose approximately 50 per cent of the theoretical amino nitrogen content, instead of the 65 per cent used in other calculations. If the former value is assumed correct for the insulin hydrolysate, the glutamic acid content would become 21 instead of 19 per cent. Hence it appears likely that insulin contains close to the 21 per cent glutamic acid recorded by Jensen and Wintersteiner (21). Harington and Mead's (20) estimate (30 per cent) was based on a determination of amide and free carboxyl groups, recalculation of their data gives 27 per cent, but this obviously is only an indirect approach.

The 36 per cent glutamic acid content reported for glutenin is considerably higher than that found by Jones and Moeller (17). However, our preparation was made by different methods which will be reported elsewhere. Only small amounts of gramicidin and tyrocidine were available. The tyrocidine hydrochloride contained 13.8 per cent nitrogen and 4.1 per cent chlorine compared to the 14.3 per cent nitrogen and 2.8 per cent chlorine reported by Hotchkiss (10), hence the determination can have only limited significance.

The sample of casein used had an abnormally low nitrogen content, a recalculation on the basis of 15.7 per cent nitrogen gave approximately 25 instead of 22 per cent glutamic acid. The results for gelatin and zein differed considerably from those reported by Dakin (25, 23).

SUMMARY

A method for the quantitative determination of glutamic acid in proteins is based upon a measurement of the loss in amino nitrogen occasioned by the transformation of glutamic acid to pyrrolidonecarboxylic acid at 125° and pH 3.3. The glutamic acid is approximately 92 per cent converted in 4 hours. Of a number of amino acids investigated, only cystine interferes. The magnitude of the correction necessary for cystine was determined. No evidence for anhydride formation in protein hydrolysates during autoclaving was obtained. The method is applicable to the determination of glutamic acid in samples ranging from 25 to 100 mg. of protein when the Van Slyke manometric apparatus is used. The glutamic acid content of a number of proteins was determined, most results agreed with previous

reports Insulin contained approximately 20 per cent of glutamic acid, gelatin 12 per cent, glutenin 36 per cent, chicken feathers 12 per cent, cottonseed globulin 20 per cent, and tobacco mosaic virus 17 per cent

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STUDIES ON RATES OF EXCHANGE OF SUBSTANCES BETWEEN THE BLOOD AND EXTRAVASCULAR FLUID

II THE EXCHANGE OF SODIUM IN THE GUINEA PIG

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The rate of exchange of the water of the guinea pig's blood with extravascular water has been determined, with deuterium oxide as the tracer substance (1). The present series of experiments has as its objective an evaluation of the rate of exchange of the sodium of the guinea pig's blood plasma with extravascular sodium, radioactive sodium (Na^{24}) has been used as the tracer material. Variations in the concentration of intravenously injected Na^{24} in the plasma have been followed with respect to time, and the rate of change used to evaluate the rate of passage of Na from the plasma to the extravascular fluid on the assumption that there is no separation of isotopes.

Ten adult guinea pigs were used for the experiments. Samples of blood were obtained from a cannula placed in the proximal end of a carotid artery. A part of the experimental data has previously been published and analyzed from a different point of view (2).

The method of measuring radioactivity (2) and the mixing time of a substance introduced by intravenous injections into the guinea pig have also been given (1). The latter evidence indicates that a foreign substance introduced intravenously in the guinea pig is at least 95 per cent mixed with the plasma at the end of 1 minute, and completely mixed in 3 minutes after injection. To allow for mixing, the several measurements in our series taken prior to 15 minutes after injection were excluded from the determination of rate of transfer of Na^{24} from the plasma to extravascular fluid.

The measurements for the different animals are given in Table I. In order to place them on a common basis, all the experiments were adjusted to the same initial concentration in the plasma. Observations with the dye T-1824 gave an average plasma volume in the guinea pig of 43 cc per 100 gm of body weight (1). This value, the weight of the animal, and the known amount of Na^{24} injected permit the calculation of the initial concentration of Na^{24} in the plasma for each experiment on the assumption

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of uniform distribution. The initial concentrations varied from 126 to 5341 β -rays per second per cc of plasma. These were all adjusted to an initial concentration of 1000 β -rays per second per cc.

TABLE I
Change of Concentration of Na^{24} in Plasma, with Time

Experiment No	Na^{24}Cl injected	Weight of animal	Time after injection	Concentration of Na^{24} in plasma	Concentration Na^{24} in plasma adjusted to initial concentration of 1000
	β rays per sec	gm	min	β rays per cc per sec	β rays per cc per sec
1	22,900	700	4 0	208	273
			7 0	177	233
			12 5	134	176
			29 3	131	172
2	25,800	700	3 0	197	230
			5 8	174	203
			11 3	153	179
			26 5	147	172
3	14,100	590	4 5	130	234
			9 3	100	180
			21 3	99	178
			66 5	90	162
4	5,240	420	4 0	74	255
			21 0	64	221
			50 0	60	207
5	34,700	750	3 3	245	228
			10 0	192	178
			18 0	191	178
			31 0	179	166
6	32,350	695	2 8	288	266
			7 0	246	227
			15 5	218	201
			25 5	207	191
			38 0	188	174
			62 0	213	197
7	12,600	530	1 2	739	1336
			7 5	124	224
8	3,130	580	1 3	86	683
			6 0	34	270
			33 0	22	175
9	15,150	600	2 3	164	279
			4 0	135	230
			12 9	92	157
10	186,500	812	1 3	2199	412
			2 1	1616	303
			3 2	1517	284
			4 2	1409	264
			78 6	933	175

The change of concentration of Na^{24} in the plasma with respect to time is shown in Fig 1. To derive the rate of movement of Na from plasma to extravascular fluid by these data, assumptions are made which are strictly analogous to those used in deriving the rate of exchange of water (1). It is assumed that the amount of Na^{24} lost from the plasma per unit of time is proportionate to (1) the number of mg of Na which move from plasma to extravascular fluid per unit of time and (2) the proportion of the sodium in the plasma which is radioactive. Part of the Na^{24} which escapes into the extravascular fluid will return to the plasma, and the amount which returns per unit of time is proportionate to (1) the amount of Na which

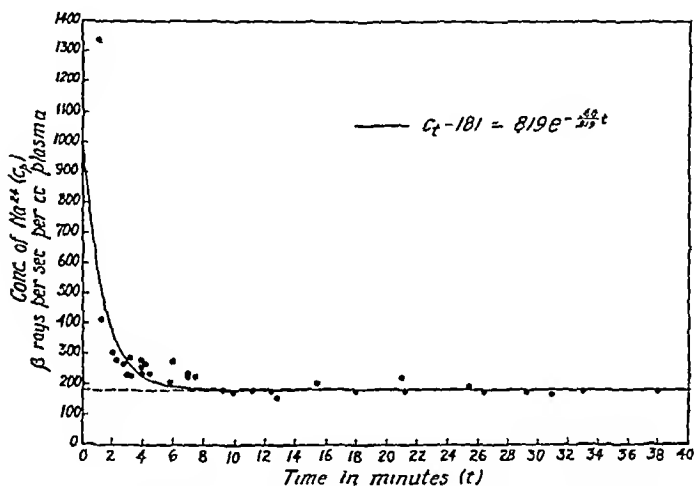


Fig 1 Change in concentration of Na^{24} in the plasma with respect to time

moves from extravascular fluid to plasma per unit of time and (2) the proportion of the extravascular sodium which is radioactive. From these assumptions, the change in amount of Na^{24} in the plasma per unit of time is given by

$$(1) \quad \frac{dN_p}{dt} = -r \frac{N_p}{Na_p} + r \frac{N_0 - N_p}{Na_e}$$

where r = mg of Na which escape from the blood vessels (or into the blood vessels from the extravascular fluid) per unit of time, N_0 = the number of units of Na^{24} in the plasma at time of injection, N_p = the number of units of Na^{24} in the plasma at any subsequent time t , Na_p = total mg of Na in the plasma, and Na_e = total mg of Na in the extravascular fluid

To express Equation 1 in terms of concentration of Na^{24} in the plasma, c_p , it is necessary to divide through by the volume of plasma, which gives

$$(2) \quad \frac{dc_p}{dt} = -r \frac{c_p}{\text{Na}_p} + r \frac{c_0 - c_p}{\text{Na}_E}$$

Integrating Equation 2 and solving for the constant of integration give

$$(3) \quad q [\ln (c_p - c_{eq}) - \ln (c_0 - c_{eq})] = -Rt$$

where $R = r/\text{Na}_p$ = the proportion of the plasma Na which escapes from the plasma into the extravascular fluid per unit of time, $q = \text{Na}_E/(\text{Na}_E + \text{Na}_p)$ = the proportion of the total sodium which is extravascular, and c_{eq} = the concentration of Na^{24} in the plasma at equilibrium

Equation 3 states that the concentration of Na^{24} in the plasma approaches equilibrium in such a way that the logarithm of the concentration in excess of the equilibrium concentration is a linear function with time. In exponential form Equation 3 may be expressed as

$$(4) \quad c_p - c_{eq} = (c_0 - c_{eq}) e^{-\frac{R}{q}t}$$

which states that the excess concentration is reduced by a constant proportion per unit of time

In Equations 3 and 4, as applied to the data of Fig. 1, c_0 is 1000 β -rays per second per cc, since the concentrations for all experiments were adjusted to this basis. Both c_{eq} and q may be derived directly from the adjusted observations after equilibrium is reached. These constants are derived as follows:

c_{eq} is obtained by averaging all of the observations at 9.3 minutes and later. These values showed no downward trend over this period of approximately 70 minutes and averaged to be 181 β -rays per second per cc. From the individual values for c_{eq} obtained from the unadjusted observations together with the known amount of radioactivity injected and the body weight, the volume available for dilution of sodium can be calculated (extracellular fluid volume). On the average, this is equal to 25.7 cc per 100 gm of body weight, with an individual variation indicated by a σ of 4.0.

Since

$$c_{eq} = c_0 \left(\frac{\text{Na}_p}{\text{Na}_E + \text{Na}_p} \right)$$

and

$$\frac{\text{Na}_p}{\text{Na}_E + \text{Na}_p} + \frac{\text{Na}_E}{\text{Na}_E + \text{Na}_p} = 1$$

then $c_{eq} = c_0 (1 - q)$

Substituting the arithmetic values of c_{eq} and c_0 , we have

$$181 = 1000 (1 - q) \text{ and } q = 0.819$$

The evaluation of these constants now makes it possible to treat the data of Fig 1 as was done with heavy water (1). If the logarithmic function of $(c_p - c_{eq})$ given by the left-hand side of Equation 3 is plotted against t , the points fall about a straight line, the slope of which is equal to $-R$, and R may therefore be determined graphically. For greater accuracy, Equation 4 was fitted by the method of least squares¹ to the observations of Fig 1 from 2.1 to 7.5 minutes inclusive. Earlier values were excluded because of incomplete mixing and the values at 9.3 minutes and beyond had already been used to determine the equilibrium value. The equation thus derived is plotted against the observations in Fig 1.

This equation gives a reasonably good description of the observations. It is worth noting, however, that there seems to be some tendency for the observations from 4 to 8 minutes to remain at a temporary level before dropping to the equilibrium value. If further observations should show that this tendency is a real factor, a more elaborate equation would be required to describe this phase of the time change. This would involve a changing rate of decline toward equilibrium instead of a constant rate, but over the major portion of the reaction, the value of the rate could not differ markedly from that obtained through Equation 4.

The value of R obtained from the fitted equation with t expressed in minutes is 0.60. This states that 60 per cent of the sodium of the plasma leaves the plasma each minute to be replaced by sodium from the extravascular source. In terms of diffusible sodium of the plasma, if we suppose that 10 per cent of plasma sodium is bound to protein (3), this means that 67 per cent of the diffusible sodium of the plasma is exchanged each minute.

The proportion of the sodium of the interstitial fluid which enters the plasma per minute is given by r/N_{aE} . This is algebraically equivalent to the expression $R(1 - q)/q$, and can be computed from the arithmetic values of R and q . This is equal to 0.132, that is, 13 per cent of the total sodium of the extravascular fluid is exchanged with the plasma sodium per minute.

Another constant of interest is the one which describes the rate at which the tracer substance approaches equilibrium. This is the exponential rate, which in the present series of experiments is equal to 0.73. This means that at any instant the rate of loss of the excess concentration above equilibrium is 73 per cent per minute.

The results of the experiments on water transfer previously reported

¹ c_p deviations weighted by the factor $(c_0 - c_p)/(c_0 - c_{eq})$ were used.

(1) may be formulated into the same series of constants just given for sodium so as to give a comparison of the rate of movement of sodium and water across the vascular membrane. This comparison is presented in Table II.

The derivation of the rate constants for water follows from those presented in the previous paper, together with certain observations on water distribution. We have shown that an exponential rate for the tracer substance of 0.82 means, in the case of water, that 73 per cent of the water of the blood is exchanged with extravascular water per minute. On the assumptions (a) that 50 per cent of the water of the blood is in the plasma, and (b) that the water of the red blood cells does not exchange directly with extravascular water, then 146 per cent of plasma water is exchanged per minute. To express the proportion of interstitial water which is exchanged per minute, we can multiply 146 per cent by the ratio of plasma

TABLE II

Constants Describing Rates of Exchange of Sodium and of Water between Vascular and Extravascular Systems

Constants	Sodium	Water
1 Proportion of amount in plasma transferred to extravascular fluid per min	0.60	1.46
2 Proportion of amount in interstitial fluid transferred to plasma per min	0.13	0.29
3 Loss in plasma concentration of tracer substance per min relative to excess concentration (exponential rate)	0.73	0.82

water to interstitial water computed from the estimates of plasma volume (4.3 cc per 100 gm of body weight) and extracellular fluid volume (25.7 cc per 100 gm of body weight), since r/w_p (w_p/w_E) = r/w_E , where w_p and w_E are respectively the volumes of water in the plasma and interstitial fluid.

A comparison of these constants for water and sodium indicates that, relative to the amounts of the two substances present, the exchange of the water across the vascular wall is about twice as great as that of sodium. That is, the ratio of water molecules to sodium ions crossing the membrane per minute is about twice what would be anticipated from their relative concentrations in the fluid bathing the membrane.

One might expect that this appreciably greater rate of water exchange relative to that of sodium would result in a more marked difference in rate of equilibration of the two tracer substances than was actually found (Constant 3). The reason that this does not follow is that the systems

involving the two substances differ. Water is contained not only in the fluid surrounding the vascular membrane but in the various cells of the body, whereas sodium is essentially extracellular.

With the difference in these systems for water and sodium what it is, similar rates of equilibration of the tracer substances imply a much faster transcapillary exchange for water than for sodium. Conversely, only in the event that the transfer of water was much faster than that of sodium would similar rates of equilibration of the tracer substances be observed.

SUMMARY

With radioactive sodium as the tracer material, the rate of exchange of sodium across the vascular membrane of the guinea pig has been determined.

It has been found that 60 per cent of the plasma sodium and 13 per cent of the sodium in the extravascular fluid are transferred in either direction across the vascular membrane per minute.

A comparison of the rate of movement of sodium and water across the membrane has been made. The amount of water relative to that of sodium crossing the vascular wall per minute is about twice what would be expected from their relative concentrations in the plasma.

The rate of equilibration of the tracer substances in the two cases is nevertheless very similar. The fact that water is both intra- and extracellular, while sodium is essentially extracellular, affects the relative rates of equilibration, and the difference in the systems for the two substances under consideration means that similar rates of equilibration imply quite different rates of transfer across the vascular membrane.

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THE IONIC STRENGTH VALENCE OF FERROHEMOGLOBIN

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Studies on the protein nature of hemoglobins, while less numerous than those devoted to the more spectacular reactions of their iron porphyrin prosthetic groups, have nevertheless been exceedingly fruitful as a consequence of the early availability of these proteins in a purified form. With solutions of the crystalline material, Hastings (1), Cohn (2), Ferry (3), and Stodie (4) and their coworkers have investigated the effect upon the electrolyte behavior of hemoglobins of their ionic environment. These studies assume practical importance with the exploitation of human hemoglobin as a by-product of blood banking. Attempts are being continued to introduce human hemoglobin as an osmotically active colloid in the therapeutics of shock and hemorrhage. Human ferrihemoglobin solutions offer some promise as an antidote for small ionic volume, hydroacid anion poisons which combine with its prosthetic nucleus (5) as well as with those heavy metals of the "b" family of Groups I and II of the periodic system of elements (6). We may anticipate a broadening of the application of solutions of the hemoglobin derivatives in intravenous therapy, so that a further knowledge of the behavior of such solutions in physiologic and pathologic ionic environments is of considerable practical utility.

Since the postulation by Loeb (7) of the stoichiometric nature of the reactions between proteins and either acids or bases, it has been customary to regard the proteins as ampholytes which differ only quantitatively, with respect to their multivalency, from the simpler amino acids. In the case of hemoglobin this multivalency is enormous, for instance, oxy hemoglobin will accept over 30 equivalents of base without evidence of saturation as determined by conductivity titration (Table I) (more suitable in this case for the demonstration of a sudden increase in hydroxyl ion concentration on stoichiometric neutralization because of the high transport number of hydroxyl). Such an increase, were it to occur, would be masked by the logarithmically decrement potentiometric effect were pH titrations relied upon to demonstrate it). That ferrohemoglobin is not saturated with respect to its apparent base receptors at 20 iron equivalents of base may be seen from its electrometric titration curve (Fig. 1).

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The high ratio of base bound by ferrohemoglobin to its non equivalency may be explained either by a stoichiometrical combination or as adsorption. If the former is the case, we are forced to assume a degree of multivalency that carries with it certain implications aside from the strictly stoichiometrical reaction of neutralization. The multivalency of ferrohemoglobin must be reflected in the ionic strength of its solutions.

TABLE I

Effect of Alkali on Conductivity of Solutions of Dog Oxyhemoglobin

40 cc. of 0.352 mM oxyhemoglobin with varying quantities of added NaOH. No end-point is observed even after the addition of over 30 equivalents of alkali.

[Hb]	[Na]	$\frac{\text{BHb}}{\text{Hb}}$	Conductivity
<i>mM</i>	<i>mM</i>		<i>ohms</i> $\times 10^{-3}$
0.014	0.040	2.8	1.61
	0.080	5.7	2.16
	0.147	10.4	3.50
	0.240	17.1	5.76
	0.310	22.0	7.80
	0.390	27.7	10.07
	0.440	31.2	11.50*
0.028	80	2.8	2.76
	97	3.4	2.97
	117	4.2	3.29
	148	5.2	3.77
	179	6.3	4.29
	210	7.4	4.85
	246	8.7	5.44
	277	9.8	6.02
	303	10.1	6.37
	365	12.9	7.80
	435	15.4	9.52
	480	17.0	10.01
	528	18.6	11.10
	607	21.4	12.83
	670	23.6	13.80
	759	26.8	16.06
	923	32.6	19.89

* This value had fallen to 9.96 after standing 20 hours.

Since it has been impossible to demonstrate a definite stoichiometrical valence for a protein by titration, one may attempt to measure its contribution to the ionic strength of its solution and thus arrive at an ionic strength valence. It is this attribute of the ferrohemoglobinate ion which is the subject of the present investigation. For the purposes of the latter, the reductant ionic activity of ferrohemoglobin is ignored and we are restricting

the term "ferrohemoglobinate ion" to that of its protein ampholytic behavior

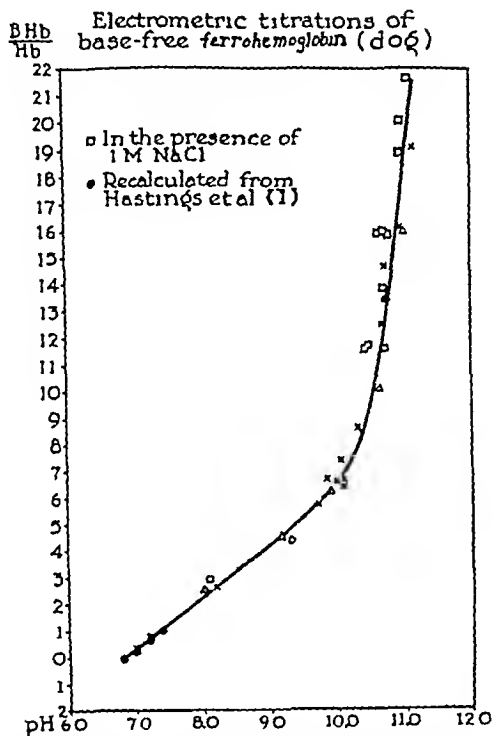


FIG 1 Electrometric titration of base-free ferrohemoglobin. Except for the points taken from the data of Hastings, Van Slyke, Neill, Heidelberger, and Harington (1) which were secured by those investigators by the CO_2 titration method, all points on this figure were from electrometric pH determinations made in the same electrode vessel as that used for the comparative titration curves. The hemoglobin solution differed from that used for the latter study in having been dialyzed free from chloride ion, so that the solutions were at the isoelectric point before the addition of alkali. These solutions were of the order of 0.1 M, so that no water correction factor was applied in the calculation of the ratio of base bound by ferrohemoglobin to the pigment, for, even assuming an enormous degree of hydration of the hemoglobin molecule, the ratio of the molal to molar concentration would still be practically unity in such dilute solutions.

When it is considered that the effect of ionic strength is in geometrically progressive proportion to the number of electronic valences, it follows that such effects in protein solutions could conceivably be massive. Stadie (8), taking into account the approximate diameter of the hemoglobin

molecule and assuming a uniform distribution of valence charges over its surface, concluded that the hemoglobinate ion would exhibit an ionic strength valence of unity. Such a value was actually secured by a measurement of the effect of the various hemoglobin derivatives on the first dissociation constant of carbonic acid.

Hastings, Van Slyke, Neill, Heidelberger, and Harington (1) found that the base bound by horse ferrihemoglobin was related to the salt concentration of the solution at a pH above that corresponding to its isoelectric point. According to the method of calculation employed in the present study, it would appear from their data that horse ferrihemoglobin combined with 2 iron equivalents of base exhibits an ionic strength valence of 2. Barnard (9) noticed that a solution of dog ferrihemoglobin produced a depression of

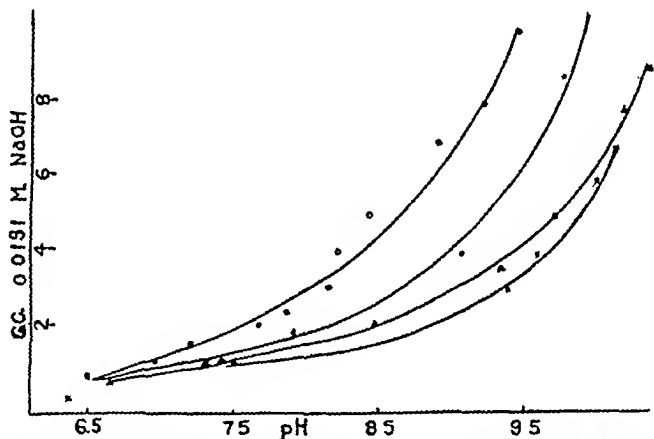


FIG 2 Titration of carbon monoxide hemoglobin $\circ, \mu = 1047$, $\bullet, \mu = 0.0646$, $\Delta, \mu = 0.0451$, $\times, \mu = 0.0303$

26 millivolts in the E_0 value of the oxidation-reduction potential of the ferricyanide-ferrocyanide system. One explanation *could* have been the high ionic strength of the protein component of the solution, and in that particular study the explanation was attributed to this.

In 1928 some preliminary comparative titrations were undertaken of solutions of carbon monoxide hemoglobin (dog) with sodium hydroxide at varying salt concentrations. Because the concentration of pigment in the solution inadvertently remained undetermined, its contribution to the ionic strength of the solution could not be calculated but it was obvious from the spread of these curves that the ionic strength valence of this hemoglobin derivative was not uniform over the titration range and must, in the vicinity of maximum spread, be greater than unity (Fig 2).

In the present investigation, the effect of the ionic strength of its solutions on the "lumped" acid dissociation constants of dog ferrohemoglobin has been studied. To this end there have been secured electrometric titration curves of the pigment from pH 6.2 to 10.7, beyond which limits the pigment is denatured.

EXPERIMENTAL

Ferrohemoglobin solutions were prepared by suction evacuation of suspensions of crystalline base free carbon monoxide hemoglobin (dog), as previously described (9). The crystals used had been subjected to but one washing, by this expedient the chloride ion associated with the pigment was not removed, thereby making it possible to observe a small portion of the acid-combining region without recourse to acid titrations. The ferrohemoglobin solutions were transferred to an electrometric titration vessel (Hastings (10)) under hydrogen and increments of standardized alkali solution (either NaOH or $\text{Ca}(\text{OH})_2$ which had been evacuated and saturated with hydrogen) were added from a micro burette. The pH was measured potentiometrically with a platinized, point platinum electrode, the chain being



The titrations were continued to the point where the solution had undergone visible conversion to globin ferrohemochromogen, this usually took place in the vicinity of pH 11. Immediately after each titration, the pH of 0.1 M HCl was determined with the same electrode that had been used for the titration. When the value obtained differed by more than 0.02 pH from the value for this normality of acid (pH 1.081), the data were discarded. All titrations were conducted at $22^\circ \pm 2^\circ$. Since the data are purely comparative, no corrections were applied for temperature, barometric pressure, water vapor content of the hydrogen, or for the E_0 of the particular calomel half-cell used. Contact and diffusion potentials were ignored (as being of insignificant magnitude) for reasons that will appear later. In spite of the somewhat diminished sensitivity of the chain (because the concentration of KCl in the agar-salt bridge had been cut down to 0.05 M to minimize alteration in the ionic strength of the solution in the titration vessel through marked diffusion of KCl into it) it was possible to run each titration rapidly, both internal and electrode equilibrium being attained within a few seconds after addition of an increment of alkali. In a few instances the contents of the titration vessel were removed for spectroscopic examination. In this way it was found that the almost indicator-like point of change from ferrohemoglobin to globin ferrohemochromogen takes place at a slightly lower pH in solutions containing sodium chloride than it does in salt-free solutions.

Calculations

The pH of the titrated solutions was calculated from the observed E_{MF} by the equation

$$pH = \frac{E_{MF} - 0.2458}{0.0591} \quad (a)$$

The theoretical ionic strength of the solutions employed was calculated from the formula of Lewis and Randall (11) adapted to these solutions

$$\mu = \frac{[Na] + [Cl] + [OH] + [Hb]z^2}{2} \quad (b)$$

If $[OH]$ is discarded as infinitesimally small and the valence of ferrohemoglobin is approximated by the number of iron equivalents of base bound by it, the completed equation becomes

$$\mu = \frac{[Na] + [Cl] + [Hb] \left(\frac{BHb}{Hb} \right)^2}{2} \quad (c)$$

The initial assumption for this approximation of the contribution of the ferrohemoglobinate ion to the ionic strength of its solutions is defensible only as an expedient. Stadie (8) deliberated this point in his paper and finally decided to treat the hemoglobin derivatives as though they were univalent electrolytes. It may be remarked that the results on the ionic strength valences finally calculated are found to be essentially the same whether the present method (i.e., in which the ionic strength contribution of the ferrohemoglobinate ion is taken as that of its apparent stoichiometrical valence) is employed or that of Stadie, in limiting this to unity, or, for that matter, one in which any possible contribution of the pigment toward the ionic strength valence of its solution is ignored in its entirety throughout the titration range (as occurs automatically in the present method of calculation in which this is made for the vicinity of the isoelectric point of ferrohemoglobin). We are faced by three methods in this matter, the last is the simplest expedient but obviously not a defensible one because the pigment does contribute to the ionic strength. From the data of Hastings *et al.* (1) obtained by the very accurate CO_2 titration method, it is seen that this contribution may be greater than unity. The present method was adopted therefore as being, theoretically at least, the correct one. The fact that there is little difference in the end-result as to what is accepted as the valence of ferrohemoglobin for this purpose finds its corollary in the inconsequentiality of whether, for the same purpose, the molecular weight of ferrohemoglobin is taken as equal to iron equivalency or as 4 times this amount. Stadie used the former figure and we have also because relatively

dilute solutions of hemoglobin may actually be dissociated into submolecules (12)

That the resultant ionic strength of the ferrohemoglobin solutions may not be unduly high is evidenced by a consideration of Curve B, Fig 3. The slope represents an adaptation of the limiting equation later developed, $\Delta pH = 2.9 \Delta \sqrt{\mu}$, β in this case having a value of 2.9. While this value corresponds to an ionic strength valence of 2.4, it is noticed that the actual slope, as indicated by the last three experimentally determined points on it, shows a tendency to level off and this occurs at the very vicinity of ionic strength which corresponds to that designated for such a departure by the

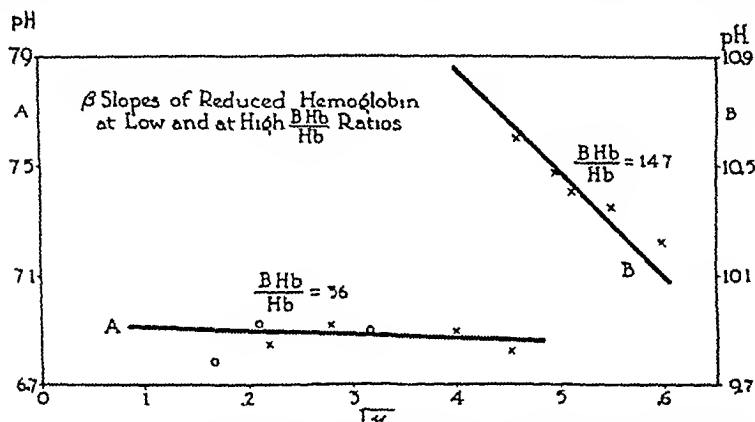


FIG 3 Titration of ferrohemoglobin solutions of constant base ratios with sodium chloride. Curve A exhibits a β slope of 0.25, while that of Curve B has a value of 2.9. The approximate linearity of the experimental points indicates that the area of the curve plotted from the data of Table II would be the segment of a flat plane. The variation of pH induced by the addition of NaCl cannot be entirely due to changes in liquid junction potential and the variation of β which has a slope over 10 times as great in Curve B as that of Curve A cannot be due to change in the dielectric constant of the medium.

expanded Debye-Huckel equation (13). The regular method applied, however, to the determination of the ionic strength valence is by calculation from the more simple limiting law derived by Debye and Huckel by adaptation of the classical three-halves power law of Poisson's to the electrolytic behavior of solutions. This limiting expression,

$$-\log_{10} f = \frac{\alpha}{2} z^2 \sqrt{\mu} \quad (d)$$

where $\alpha = N^2 e^3 2 / (10 R D_0 T_0)^{1/2}$, all of whose terms are universal constants, has been simplified by Bionsted and La Mer (14) who express the left-hand member in positive notation and combine α and z into a single series of

constants, β , whose value is a geometric function of the valence type, while μ , as in the limiting equation, is the ionic strength as defined by Lewis and Randall (11)

It is necessary for an experimental evaluation of the ionic strength effect to measure the change in the activity of a constituent of the solution. Such a change would be geometrically proportional to the ionic strength valence and theoretically the method would be more accurate for a designation of the higher valence types.

As the index to the change in ionic strength, Stadie and Hawes (4) used for the criterion the activity of bicarbonate ion. This method is complicated by the unknown extent to which the carbamino reaction concurs. It has been deemed advisable to use, instead, the apparent activity of the ferrohemoglobinate ion itself.

Because ferrohemoglobin has at least twenty acidic groups each with its individual dissociation constant and because of the hopelessness of any attempt to make an isolated measurement of any one of these, we may assume that, as in the cases of all other polyvalent anions, the individual dissociation ranges are not coextensive. In the case of ferrohemoglobin we may likewise assume, as did Stadie and Martin (15), that these individual dissociation constants can be treated as though they were spaced at more or less equal intervals throughout the pH scale. This assumption finds some support in the absence of gross inflections from the titration curve of ferrohemoglobin.

If, then, we titrate a solution of ferrohemoglobin with alkali, we may consider that at any particular portion of the curve we are in the vicinity of the half neutralization point of some particular acidic group and by application of the Henderson-Hasselbalch equation to this group,

$$\text{pH} = \text{pK}_s - \log \frac{[\text{HA}_s]}{[\text{BA}_s]} \quad (e)$$

In the defined vicinity where

$$[\text{HA}_s] \cong [\text{BA}_s], \quad \log \frac{[\text{HA}_s]}{[\text{BA}_s]} \rightarrow 0 \quad (f, g)$$

$$\text{pH} = \text{pK}_s \quad \text{and} \quad \Delta \text{pH} = \Delta \text{pK}_s \quad (h, i)$$

Since the limiting law (d) is a linear equation,

$$\text{pK}_s = \beta \sqrt{\mu} \quad \text{and} \quad \beta \text{ is a constant} \quad (j)$$

$\Delta \text{pK}_s = \beta \Delta \sqrt{\mu}$ and if we substitute from (i)

$$\beta = \frac{\Delta \text{pH}}{\Delta \sqrt{\mu}} \quad (k)$$

At 22°, β is almost exactly 0.5. The ionic strength valence may therefore be calculated

$$V_i = \sqrt{2\beta} \quad (1)$$

The foregoing is based on the classical dissociation theory of ampholytes and ignores the fact that ferrohemoglobin, like other proteins, is a massive

TABLE II

Effect of Ionic Strength on Acidity of Dog Ferrohemoglobin

Titration of 10 cc. of 1.44 mM dog ferrohemoglobin with 31.4 mM NaOH in the presence of NaCl and NaBr. pH 0.1 M HCl 1.079. Concentrations in mM per liter

Hb	Na	Halide	$\frac{BHb}{Hb}$	pH (chloride)	pH (bromide)	$\sqrt{\mu}$
1.44	0.0	2.0	-1.24	6.089		0.0464
1.32	3.0	1.9	0.87	7.214		0.0500
1.20	5.0	1.8	3.02	8.051		0.0945
1.12	8.0	1.7	4.80	9.140		0.1311
1.05	10.0	1.5	6.67	9.911		0.1710
0.90	15.0	1.3	11.90	10.580		0.2690
0.80	18.0	1.1	16.10	10.861		0.3370
0.75	19.0	1.0	18.35	10.921		0.3690
1.44	91.0	92.8	-1.24	6.210	6.259	0.3029
1.40	90.0	88.4	0.22		6.693	0.2971
1.31	87.0	82.8	1.24	7.169	7.201	0.2930
1.20	84.0	77.6	3.00	7.643		0.2940
1.11	80.0	77.2	5.01	8.231		0.2998
1.05	77.0	68.8	5.80		9.216	0.3040
0.96	76.0	66.4	7.47	9.752		0.3120
0.94	74.0	64.2	8.00		10.004	0.3153
0.92	72.0	62.0	9.61	10.119		0.3307
0.90	70.0	57.0	11.90	10.289	10.321	0.3569
0.80	65.0	51.6	16.10	10.630		0.4020
0.75	63.0	49.0	18.35	10.786		0.4300
0.72	62.0	46.5	20.24	10.962		0.4511

* Calculated from the added base after subtraction of the amount found necessary to bring the solution to the isoelectric point (pH 6.810)

and complex zwitter ion. Far from imposing any difficulties, the concept of the zwitter ion complex assists in explaining the irreconcilability of the calculated and measured ionic strength valences of ferrohemoglobin, while it does not affect our method of calculation of the shift in the lumped dissociation constant on which the experimental values are based.

Results

Table II represents a titration of ferrohemoglobin in salt-free solution and its comparative titration in the presence of sodium halide. The β

values calculated from this experiment are given in Table III. It is apparent that the ionic strength effects of sodium chloride and sodium bromide are practically identical, as would be predicted by the Debye-Huckel

TABLE III
Effect on Its Ionic Strength of Sodium Bound by Ferrohemo globin

$\frac{\text{BHb}}{\text{Hb}}$	ΔpH	$\Delta\sqrt{\mu}$	β
-1.24	0.132	0.256	0.515
2.0	0.25	0.22	1.13
4.0	0.65	0.18	3.61
6.0	0.45	0.14	3.21
8.0	0.27	0.11	2.46
10.0	0.28	0.10	2.80
12.0	0.28	0.085	3.03
14.0	0.22	0.075	3.41

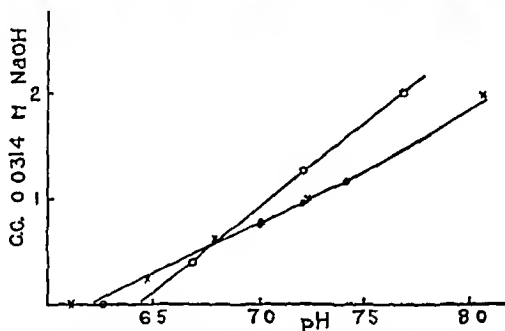


FIG 4 Microtitration of 10 cc of 1.9 mM ferrohemoglobin with 0.0314 M NaOH. X, salt-free ferrohemoglobin, ●, recalculated from Hasting *et al* (1), O, ferrohemoglobin in 0.1 M NaCl. This experiment appears to justify the assumption that liquid junction potentials in the electrometric chain are not responsible for any large error in the determination of pH in ferrohemoglobin solutions nor is a variation of liquid junction potentials the cause of the shift in the pH of such solutions at differing salt concentrations. It is observed (a) that the isoelectric point of ferrohemoglobin coincides with that arrived at by cataphoresis and solubility experiments and (b) the effect of similar concentrations of added NaCl on the observed potential is approximately equal and opposite in sign at opposing equidistant directions from the isoelectric point.

theory. It is also to be noted that each salt exerts an effect upon both the acid- and base-combining powers of ferrohemoglobin everywhere on the titration curve (except, of course, at the isoelectric point of the pigment). This effect is to increase the buffer power of ferrohemoglobin

The relative absence of salt effect near the isoelectric point is brought out by a consideration of Fig 3, in which is represented a comparative titration of ferrohemoglobin solutions of constant base content with

TABLE IV
Calcium and Sodium Combining Powers of Dog Ferrohemoglobin
Concentrations in mm per liter

A 10 cc 0.581 mm ferrohemoglobin titrated with 0.04 M NaOH and 0.02 M Ca(OH) ₂ pH 0.1 M HCl 1.087						B 10 cc 1.90 mm ferrohemoglobin titrated with 0.04 M NaOH and 0.02 M Ca(OH) ₂ pH 0.1 M HCl 1.092					
[Hb]	[Na]	[Ca]	BHb/Hb	pH	$\sqrt{\mu}$	[Hb]	[Na]	[Ca]	BHb/Hb	pH	$\sqrt{\mu}$
0.565		1.24	1.34	7.387	0.0552	1.90		0.0	-1.65	6.212	0.064
0.562		1.44	1.69	7.698	0.0607	1.89		0.30	-1.57	6.225	0.068
0.556		1.62	2.07	8.051	0.0668	1.86		0.69	-1.37	6.289	0.069
0.555		1.80	2.36	8.301	0.0719	1.84		1.22	-1.11	6.471	0.071
0.550		2.08	2.98	8.985	0.0815	1.81		1.80	-0.77	6.562	0.076
0.548		2.30	3.34	9.316	0.0886	1.77		2.48	-0.37	6.732	0.081
0.545		2.47	3.71	9.569	0.0935	1.76		3.61	-0.26	6.915	0.086
0.540		2.78	4.27	9.766	0.1020	1.69		3.79	-0.37	6.994	0.095
0.535		3.00	4.75	9.952	0.1080	1.60		5.19	1.53	7.339	0.117
0.530		3.60	5.96	10.191	0.1290	1.53		7.10	2.78	7.941	0.148
0.531			-0.09	6.207		1.49		8.59	4.05	8.035	0.163
0.578		0.35	-0.02	6.632	0.0274	1.46		9.27	4.57	8.283	0.190
0.575		0.50	0.00	6.821	0.0321	1.38		10.38	5.61	8.479	0.222
0.570		0.75	0.52	7.069	0.0398	1.34		11.91	7.20	9.229	0.240
0.565		1.13	1.20	7.363	0.0518	1.30		12.42	7.34	9.530	0.254
0.552		1.88	2.58	8.404	0.0749	1.27		13.40	8.79	9.739	0.280
0.550		2.08	2.90	8.833	0.0805	1.21		15.10	10.90	10.112	0.324
0.505		5.25	9.49	10.662	0.1820	1.14		16.64	12.35	10.362	0.346
0.581			-0.09	6.193		1.10		17.88	15.10	10.578	0.406
0.573	0.60		0.17	6.913	0.0187	1.75	3.82		0.37	7.089	0.059
0.564	1.21		1.23	7.541	0.0324	1.61	6.28		2.07	7.618	0.089
0.552	1.92		2.56	8.753	0.0529	1.58	6.93		2.47	7.802	0.092
0.550	2.11		2.87	9.071	0.0579	1.49	9.29		4.57	9.032	0.150
0.540	2.81		4.24	9.856	0.0791	1.40	10.37		5.61	9.491	0.159
0.530	3.60		5.83	10.214	0.1031	1.38	12.35		7.26	9.848	0.204
0.520	4.64		6.52	10.332	0.1140	1.29	14.44		8.79	9.990	0.242
						1.20	14.98		10.80	10.389	0.290
						1.09	17.10		13.25	10.541	0.346
						1.02	18.42		16.10	10.670	0.374

varying amounts of sodium chloride. The slope at BHb/Hb = 0.36 is almost horizontal, at BHb/Hb = 14.7 the slope is almost 3, which corresponds to an ionic strength valence of about 2.5. This value agrees with that derived from Table III for this particular base ratio and is prob-

ably close to the apparent maximum ionic strength valence demonstrable by calculation from (I) ¹

Fig 4 represents a titration of ferrohemoglobin in the vicinity of the isoelectric point, in the presence and in the absence of added sodium chloride. It shows that the salt effect at $HbX/Hb = BHb/Hb = 1$, where the ionic strength valence is unity, is in accordance with the value arrived at by Stadie (whose data were confined to the acid side of the curve), because the ΔpH values are almost identical, though opposite in sign, for the left-hand and middle members of the equation. Since at the point of the right-hand equality the ionic strength value is unity, this must be true of the left-hand member as well.

Table IV gives the results of comparative titrations of ferrohemoglobin with sodium and calcium hydroxides. Curves plotted from Table IV (not reproduced) show a similarity in contour but throughout the titration

TABLE V
Effect on Its Ionic Strength of Calcium Bound by Ferrohemoglobin

Experiment A, Table IV				Experiment B, Table IV			
$\frac{BHb}{Hb}$	ΔpH	$\Delta\sqrt{\mu}$	β	$\frac{BHb}{Hb}$	ΔpH	$\Delta\sqrt{\mu}$	β
1	0.15	0.0180	8.3	0.37	0.095	0.034	2.79
2	0.34	0.0190	18.2	4.57	0.749	0.040	18.21
3	0.34	0.0180	18.9	5.61	1.012	0.063	16.09
4	0.21	0.0191	10.9	7.20	0.611	0.036	16.94
5	0.13	0.0210	6.2	8.79	0.251	0.038	6.61
6	0.52	0.0222	23.6	10.90	0.259	0.032	8.09

more calcium than sodium equivalents are bound by ferrohemoglobin. The values calculated from these experiments (Table V) are of the order of magnitude predictable by the Debye-Huckel theory, though inordinately high for calcium ion. The explanation may reside either in a specific ion effect or in a lesser degree of dissociation for calcium than for sodium ferrohemoglobinate.

DISCUSSION

For the purposes of the investigation it is necessary to evaluate the factors which will affect the veracity of the value β as determined, and to

¹ In this connection it is pointed out that even ions of a simple valence type may exhibit β values slightly lower than would be calculable from equation (I). Thus for the pK_2 of carbonic acid, Hastings and Sendrov (16) obtained the value $\beta = 1.6$ instead of 2. For the pK_3 of orthophosphoric acid, Sendrov and Hastings (17) obtained a β value of 4 instead of the theoretical 4.5.

outline the difficulties in the interpretation of the results once it has been admitted that the slope β actually represents a function of the ionic strength valence of ferrohemoglobin. In the first category there are those factors which affect the accuracy of the determination of pH of protein solutions from electrometric data. The assumptions already made in this connection by Stadie are considered to be sound both from the reasoning presented by that investigator and by evidence uncovered during the course of the present investigation. The three factors in this category are (a) insignificance of liquid junction potentials, indicated in this study by the coincidence of the isoelectric point of ferrohemoglobin from electrometric data at different salt concentrations which corresponds to the pI as determined by methods of CO_2 titration employed by Hastings *et al.* (1), (b) the insignificance of hydration effects is an assumption even more valid in the present work than in that of Stadie's because of the high ratio of solvent to solute employed in the former, and (c) absence of irreversible reactions in the system $\text{B}^+ + \text{X}^- \rightleftharpoons \text{Hb}^+ + \text{OH}^-$ for which the absence of evidence of spectroscopic alteration until the point of ferrohemochromogen formation is reached can be considered a reasonable indication.

From equation (4), assuming that the validity of the numerator of the right-hand member is thus established, the veracity of calculated β is dependent upon $\Delta\sqrt{\mu}$. It is significant in this regard that, while the legitimacy of the calculations of the absolute value of μ is in some doubt (because of the assumptions made in taking the molecular weight of ferrohemoglobin as that of its iron equivalency and its ionic strength contribution as a geometric function of its base ratio), the value of $\Delta\sqrt{\mu}$ is not thereby called to question. The latter is a linear function of a uniform slope, and herein lies the explanation of the paradox that it is immaterial to the final result what value is assigned to the stoichiometric valence of ferrohemoglobin for the calculation of the ionic strength of its solutions, for in the ultimate step, the calculation of $\Delta\sqrt{\mu}$, the square root is taken of a square law function and the result is bound to approximate one of the first order. Likewise for the numerator of the equality (4), ΔpH , the expedient of its use relegates the question of the absoluteness of pH values to the background. Actually it may not connote a change in the activity of the acidic groups at all, for according to the zwitter ion concept what has been measured is a change in the apparent basic dissociation constants. Which actually have been measured is of little consequence in so far as they concern the inquiry into the ionic strength valence.

The interpretation of the variations in β must, from the fundamental equation and from the experimental conditions imposed, reflect either a change in the dielectric properties of the solvent induced by the solutes or a variation in the ionic strength valence of ferrohemoglobin. These two

possibilities will be discussed before the only alternative explanation is brought up that the Debye-Huckel theory may not be applied without reservation to solutions containing dipolar ions

Dielectric Constant of Ferrohemoglobin Solutions—The dearth of data as to changes in the electrostatic permeability of solvents induced by simpler ions, let alone protein ions, is, as Stadie has pointed out, the largest stumbling block in the unreserved application of the Debye-Huckel theory to such solutions. There is in addition the further consideration, not properly appreciated at the time of Stadie's analysis, that ferrohemoglobin solutions are paramagnetic (18, 19). One may debate the propriety of referring to a dielectric *constant* of a paramagnetic medium at all, but there are heuristic arguments for the acceptance of the position that the concentration of ions extant in the medium in the present and in Stadie's study is without appreciable effect on the electric parameters of this medium. An attempt was made to measure any effect of ferrohemoglobin on these parameters with negative results. The method used is open to the criticism of insensitivity but it is the best available for the purpose at hand.

For the absolute determination of the dielectric constant of a medium throughout which ionic forces are at play, it would be necessary to measure the velocity through this medium of radiant energy of such frequency that its wave-length would be small in comparison to the distance between the ions. If the dimensions of the medium are massive in comparison to those of the artifacts within it, the over-all permeability and susceptibility may, for practical purposes, be taken as equal to that of the medium itself. Thus Maxwell's field equations are applied to air as though it were free space, notwithstanding the fact that its contained oxygen is definitely paramagnetic and in spite of the fact that these equations break down completely when we get down to dimensions far above those of the gas molecules in air. This is so because for those wave-lengths appreciably above the dimensions of artifacts in an absolutely heterogeneous field the latter becomes homogeneous. We cannot hope to use any radiant energy practically available for an absolute determination of dielectric constant in a heterogeneous field and thus we cannot determine the dielectric constant of the medium in the immediate vicinity of valence charges. It is the last parameter that may be the all important one. For the present, however, we have no alternative but to treat the solutions employed in this study as an isotropic homogeneous medium and to measure the effect of ferrohemoglobin on the apparent dielectric constant of a sodium chloride solution of the same order of concentration as those employed in securing the comparative titrations. This was done by the measurement of its effect on the distributed capacity of the inductive component of an antiresonant electrical circuit. The frequencies used were as high as was compatible with accuracy in measure

ment and too high for the measurements to have been thrown off by the effect of ionic mobilities

For this purpose the Boonton Q meter was employed. The apparatus consists of a variable high frequency oscillator supplying the antiresonant circuit, the potentials across the latter being read in terms of Q on a vacuum tube potentiometer. The inductance consisted of a coil comprising six turns of No. 12 enameled copper wire wound snugly around a $6 \times \frac{5}{8}$ inch soft glass test-tube in which was placed the solution whose comparative dielectric constant was to be determined. The circuit was brought into antiresonance by adjustment of the variable condenser in parallel with the inductance, for different frequencies between 12 and 45 megacycles, in the presence and absence of ferrohemoglobin in the solution. There was no

TABLE VI

Effect of Ferrohemoglobin on Apparent Specific Inductive Capacity of Sodium Chloride Solution

$\omega \times 10^6$	0.11 mM NaCl		0.18 mM ferrohemoglobin in 0.11 mM NaCl		Distributed capacity*	
	Q	C^*	Q	C^*	With ferrohemoglobin	Without ferrohemoglobin
12			203	446.11	0.50	
15	231	290.49				0.51
18			243	202.40	0.60	
25	258	101.68	255	101.63	0.60	0.55
35	216	50.55				0.56
36.9			200	45.70	0.56	
45	144	29.91	148	29.91	0.615	0.615

* In micromicrofarads

significant difference in the readings obtained from the two solutions (Table VI). Apparently the presence of ferrohemoglobin in the concentration of 0.1 mM is without effect on the over-all dielectric constant of sodium chloride of like concentration *for this particular band of frequencies*.

The values of Q observed in this experiment are also given in Table VI. Ferrohemoglobin as a ferromagnetic substance should have exhibited for the solution in which it was incorporated a slightly higher Q value than that of the solution from which it was absent. (Actually this is seen to be the case when Q/λ is plotted, the point reversal at 25 megacycles is undoubtedly an experimental error.) The method is not, however, devised for the accurate determination of magnetic susceptibilities. But the coincidence of the Q values has a more pertinent significance. While Q is ordinarily used as a "figure of merit" for inductances and capacitances, we

may adduce from the fact that there is little difference in its value with or without ferrohemoglobin an additional proof that the latter is without marked effect on the dielectric constant of its solutions at any wave-length. For

$$Q = \frac{\omega L}{R'} = \frac{1}{\omega C R'} \quad (m)$$

$$Q^2 = \frac{L}{C R'^2} \quad (n)$$

and

$$Q = \sqrt{\frac{L}{C}} \frac{1}{R'} \quad (o)$$

the frequency terms disappearing and Q having the dimensions of an impedance. Now the square root of the ratio of magnetic susceptibility to dielectric constant is likewise an impedance and a constant one for any isotropic, homogeneous medium in which a fixed velocity of propagation of electromagnetic energy obtains. Pauling and Coryell (19) have measured the magnetic susceptibility of ferrohemoglobin solution and have found it to be constant over a pH range coinciding with that covered in this study. Since magnetic susceptibility is inductance per unit distance, it necessarily follows that the dielectric constant which is capacitance per unit distance must likewise be uniform over this range. The varying β values, therefore, could not, by this reasoning, be due to change in dielectric constant of the solutions with varying hydrogen ion concentration.

Calculations from the data shown in Fig. 3 likewise serve as an indication to construe these varying β values as due to something other than a change in the dielectric constant of the medium, exclusively. Unless some other construction is put upon them, one cannot escape some rather remarkable conclusions. If we assign an ionic strength valence of unity to ferrohemoglobin, from the equation for α , the β slope, Curve A in Fig. 3, would connote a dielectric constant higher than that of any known substance, while from the β slope, Curve B, there would be indicated for the solution a dielectric constant half that of free space. The speed of light through such a medium would be 4 times that in a vacuum.

It is thus probable that variations in exhibited β value are not conditioned by dielectric changes in the medium. Suggestive evidence that they should not be so construed is also yielded from Stadie's data. Nitric oxide and carbon monoxide ferrihemoglobin were found by Stadie to lower the β value in the activity equation as applied to bicarbonate ion, while ferrohemoglobin, ferrihemoglobin, and cyanide ferridehemoglobin did not affect the theoretical value for a univalent ion markedly. A more likely explana-

tion than that the ferrylhemoglobins produced a marked rise in the dielectric constant of their aqueous solutions is that, their isoelectric point being closer to the pH corresponding to the pK_1 of carbonic acid, their ionic strength valence in this range is less than unity. That of ferrohemo- globin and ferrihemoglobin (and probably the ferridehemoglobins), having isoelectric points further removed from this vicinity, exhibit a normal β value for a univalent ion because, fortuitously enough, the pI of the latter is just such a distance from the pK_1 of carbonic acid that the pigments have a stoichiometrical valence of 1 at the latter point.

Interpretation of β Slope for Protein Solutions—In the face of the results of the present investigation those theoretical considerations which led Stadie (8) to ascribe a maximum ionic strength valence of unity to ferrohemo- globin are untenable. They would unquestionably be valid if the molecule were a globular mass with the valence charges distributed uniformly over its surface, each charge might then be sufficiently removed from its neighbors to be considered as outside their electrostatic spheres of influence. The probabilities are, however, that these valence charges (some at least) are within the body of the molecule and, therefore, if the distribution is uniform or otherwise, closer together than could possibly be the case with a uniform surface distribution. Many charges would be likely to fall within the activity spheres of others and the molecule to exhibit ionic strength polyvalency. For it cannot be held that valence charges within a protein molecule are effectively removed from participation in the ionic environment of its solution, stoichiometrical behavior negates such an assumption. The protein molecules have enormous lattice constants and the diffusion rate of smaller ions through the interstices of the solvated protein molecule is not of a different order of magnitude than this rate through the medium of solution.

If the variations in β are then to be interpreted as conditioned by a variation in the ionic strength of ferrohemo- globin, what coefficient are we to set in defining this function? With the ordinary value set for β , the maximum ionic strength valence of ferrohemo- globin is far below its stoichiometrical valence at the position where the former is at a maximum. One explanation could actually be inherent in the Debye-Huckel theory as elaborated. The theory was not designed for unreserved application to solutions of dipolar ions but qualification of the theory for application to such solutions should be possible. We may do this tentatively and arrive at an explanation of the qualitative phenomena as observed in ferrohemo- globin solutions. At the isoelectric point, inner salt formation would be complete, and c in the expression for α would be zero (because of the absence of electrostatic charge on the molecule). As the titration is carried away from the isoelectric point, dipoles come into existence in a number

proportional to the evinced stoichiometrical valence, but the elements of these dipoles may not each exert a unit electrostatic charge because of random distribution and neutralization due to proximity. The effect would be one of the reduction in net electrostatic charge. This means that the discrete values of β would be correspondingly reduced for each degree of connoted ionic strength valency. This explanation is out of line with what has been observed in titrations performed on glycine. The latter are represented in Table VII. The maximum β value, which, as in the case of ferrohæmoglobin occurs near the mid-point of neutralization, is abnormally high for a divalent ion, at other portions of the curve the value is orthodox. Thus for a solution of this dipolar ion we face a situation in which the explanation of the abnormally low β values as observed in ferro-

TABLE VII

Effect of Ionic Strength on Apparent Acid Dissociation Constant of Glycine (Calculated on Basis of Classical Theory)

Log $\frac{\text{Na glycinate}}{\text{glycine minus glycinate}}$	pH	pK _a	pK _c	$\sqrt{\mu}$	β
-0.784	8.522	9.304	9.662	0.237	1.49
	8.301	9.084	9.669	0.384	
-0.404	8.859	9.264	9.780	0.219	2.40
	8.535	8.944	9.788	0.352	
-0.125	9.041	9.166	9.831	0.208	3.19
	8.647	8.774	9.830	0.329	
0.125	9.119	8.995	9.745	0.200	3.73
	8.701	8.570	9.745	0.312	
0.399	9.210	8.811	9.791	0.183	1.90
0.785	9.330	8.546		0.179	
	9.136	8.368		0.279	

hemoglobin solutions does not hold unless we made the further qualification that glycine, in the vicinity of the half neutralization point of its acidic group, exhibits more than divalency (by hydration of the carbonyl?), a possibility which is highly remote. The results with glycine dictate caution in the interpretation of the absolute magnitude of β in dipolar ion solutions at the present state of knowledge. That the variations in this value are indicative of variations in ionic strength valence can hardly be doubted, the question of the magnitude of the latter variations should be left open.

Practical Considerations from Effect on Acidity of Ferrohæmoglobin of Its Ionic Environment—The increase of acidity of a protein on the basic side of its isoelectric point is an effect qualitatively predictable from the Debye-Huckel theory. This increase would be greater in degree for plasma proteins than for ferrohæmoglobin in which the effect is considered within the

physiologic pH range, the former would be farther removed from their isoelectric points. Whether the behavior of ferrohemoalbumin is duplicated by plasma proteins and whether the magnitude of salt effect is physiologically sensible are questions which merit further inquiry.

From the absence of ionic strength effect of ferrohemoalbumin at its isoelectric point, its solubility at this point should be at a minimum. While there are no data on the reduced form of the pigment, it has been observed during its preparation from suspensions of carbon monoxide ferrihemoalbumin that, when the latter had been washed free from all salt at its isoelectric point, it was practically insoluble and barely tinged the water in which the crystals were suspended.

TABLE VIII

$\Delta E_0/\Delta pH$ Slope of Horse Ferrihemoalbumin-Ferrohemoalbumin System (Recalculated from Conant (20))

Sample	pH (assumed)	pH (corrected)	E_0	$\frac{\Delta E_0}{\Delta pH}$
A	6.42	6.50	-0.123	
	8.44	8.17	-0.221	0.059
	9.42	9.08	-0.244	0.025
				0.042
B	6.42	6.50	-0.116	
	8.44	8.10	-0.179	0.039
	9.42	9.00	-0.252	0.081
				0.060
C	6.42	6.50	-0.103	
	8.44	7.96	-0.183	0.055
	9.42	8.92	-0.270	0.087
				0.071

The buffering activity of ferrohemoalbumin is practically linear and uniform through its isoelectric point and the effect of an increase in the ionic strength of its solution is to increase the buffering power without affecting the uniformity of the buffer slope. The increased buffer power of ferrohemoalbumin (and ferrihemoalbumin, which appears to have an identical degree of base receptivity) must be taken into account in pH studies in which protein is incorporated into non-protein buffer solutions, for, even though the relative molar concentration of the former appears to be slight, it may effect a considerable change in the final pH of the solution. Thus, in his determination of the value n in the equation for oxidation-reduction potential of the ferrihemoalbumin-ferrohemoalbumin system

$$E_2 = E_1 - \frac{RT}{nF} \log_e \frac{[\text{ferrihemoalbumin}]}{[\text{ferrohemoalbumin}]} \quad (p)$$

Conant (20) took the final pH of the buffer-protein mixture to be the same as that of the unmixed buffer and concluded as the result of this technical error that the value of n was higher than it was subsequently proved by others to be. But the proper value of n can also be calculated from Conant's original data when the actual pH of the buffer-hemoglobin solutions is determined by correction for the buffer value of the protein at the ionic strength of the buffers employed. When this is done (Table VIII), the value of n so calculated is in excellent agreement with that found by Havemann and Wolff (21), Taylor and Hastings (22), as well as by Barnard (9). It is worthy of note that in the last study the evaluation of pH of the hemoglobin solutions which entered into the calculation of the value of n was arrived at directly from the BHb/Hb ratios at varying ionic strength as derived from the data of the present investigation. The concordance of the value of n in the oxidation-reduction equation as determined by this means with that value arrived at by other investigators is a rigorous check on the accuracy of the experimental work of the present paper.

SUMMARY

The acidity, basicity, and buffer powers of ferrihemoglobin are increased by the addition of sodium chloride or sodium bromide to its aqueous solutions. The magnitude of this increase may be considered to be in harmony with the behavior of an ampholyte in the light of the Debye-Hückel theory.

The ionic strength valence of ferrihemoglobin is zero at its isoelectric point and rises on either side of that point to an apparent maximum of about 3 on the basic side of the titration curve.

From theoretical considerations and on the basis of direct measurement, ferrihemoglobin is without effect on the dielectric constant of aqueous solutions.

More calcium than sodium ion is bound by ferrihemoglobin at any pH above that of its isoelectric point. The magnitude of the difference between the two cations is greater than can be explained by the Debye-Hückel theory.

The calculated maximum ionic strength valence of ferrihemoglobin is much lower than its stoichiometrical valence, while that of glycine is higher than its known stoichiometrical valence. It is concluded that the Debye-Hückel theory is not to be applied unreservedly to solutions of dipolar ions.

Symbols

- [] = concentration on a molar basis
- E_0 = the characteristic voltage of a half-cell
- μ = ionic strength, one-half of the sum of the concentrations of each of the ions present times the square of its valence
- z = stoichiometrical valence

V_i = ionic strength valence

α = a constant defined by universal terms

β = a series of constants whose value is determined by that of α and the stoichiocochemical valence of the ion to which it is applied

N = Avogadro's number

e = the charge upon an electron

R = the gas constant

pK_1, pK_2, \dots = the negative logarithms of the first, second, n th dissociation constant of a polybasic acid or polyacidic base

pK = the negative logarithm of the dissociation constant of any particular acidic group in ferrohemoglobin which is near the point of half neutralization at some region of the titration range of the pigment

Q = figure of merit of an inductance or capacitance

R' = electrical resistance in ohms

L = electrical inductance in henries

C = electrical capacity in farads

ω = frequency in cycles per second

λ = wave-length

D = dielectric constant (specific inductive capacity)

T = absolute temperature

F = Faraday's electrochemical equivalent

n = the number of electrons involved in the change from oxidized to reduced form

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THE METABOLISM OF L(-)-PROLINE STUDIED WITH THE AID OF DEUTERIUM AND ISOTOPIC NITROGEN*

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The structural similarity of the 5-carbon amino acids, ornithine, proline, hydroxyproline, and glutamic acid, has provoked much experimentation and speculation as to their possible metabolic interrelationships. Dakin (1) showed that proline, ornithine, and glutamic acid yielded approximately equal amounts of glucose when given to a glycosuric dog and did not increase acetoacetic acid formation when they were perfused through dog livers. Kapfhammer and Bischoff (2) demonstrated that the ingestion of equivalent quantities of proline and hydroxyproline gave rise to the same amounts of extra glucose in the urine of phlorhizinized dogs. On the other hand, Edson (3) presented evidence for the ketogenic nature of hydroxyproline in the presence of rat liver slices, a property not shared by proline, ornithine, and glutamic acid.

The suggestion that the animal can convert glutamic acid to proline was made by Abderhalden (4) after showing that protein hydrolysates rich in glutamic acid but freed of proline by alcohol extraction were nutritionally as effective as whole hydrolysates.

The conversion of proline to glutamic acid is better established (5, 6). Such a conversion would necessarily be accompanied by the consumption of oxygen and the appearance of primary amino nitrogen. The addition of proline, and to a lesser extent of hydroxyproline, to suspensions of kidney and liver slices results in an increased oxygen uptake (7, 8) and amino nitrogen formation (5, 6). Weil-Malherbe and Krebs (5) were able to isolate α -ketoglutaric acid from the reaction products and Neber (6) obtained glutamic acid itself. Incidentally, both groups of workers ruled out pyrrolidonecarboxylic acid and Neber ruled out α -amino- δ -hydroxyvaleric acid as intermediates in this conversion by demonstrating that these compounds failed to give glutamic acid. Krebs has presented a second line of evidence based on the appearance of an increased amount of amide

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nitrogen when amino acids were added to kidney slices suspended in a medium containing ammonia. Of all the amino acids only proline and hydroxyproline behave like glutamic acid in bringing about this increase which is ascribed to the formation of glutamine. The fact that hydroxyproline gives rise to amino nitrogen less rapidly than does proline suggested to Krebs that hydroxyproline is not an intermediate in the conversion of

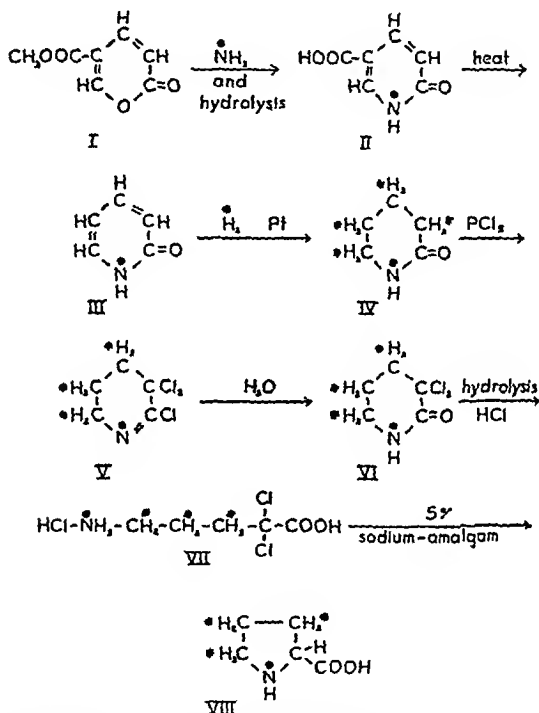


FIG 1 Synthesis of proline containing deuterium and N¹⁵ * indicates hydrogen marked with deuterium, • indicates nitrogen marked with N¹⁵

proline into glutamic acid but that perhaps a part of the hydroxyproline is metabolized by way of proline

The possibility that proline may be biologically convertible into ornithine is indicated by two lines of evidence. Ackermann (9) and Neuberg (10) have isolated both *n*-valeric and δ -aminovaleric acids from the products of the bacterial catabolism of proline. Krebs (11) has shown that extracts of mammalian kidney can oxidize optically abnormal proline and ornithine to the same keto acid, α -keto- δ -aminovaleric acid

That the intact animal can convert ornithine into both glutamic acid

and proline has been shown by work previously reported from this laboratory (12). When *dl*-ornithine labeled with deuterium was fed to mice, both the glutamic acid and the proline isolated from their bodies contained significant amounts of isotope.

To obtain further evidence respecting the possible interconversions of these compounds *dl*-proline containing N^{15} and deuterium has been synthesized by the reactions shown in Fig. 1 and the *l*(-)-proline prepared from it fed to rats. Two isotope labels were used in order to be able to follow both the nitrogen and the carbon chain of proline. A number of amino acids were isolated and the distribution of isotope in various organs and excreta was studied.

EXPERIMENTAL

Synthesis of dl-Proline with Deuterium and N^{15} —Coumalic acid, prepared by heating malic acid with fuming H_2SO_4 according to von Pechmann (13), was esterified with CH_3OH and the methyl ester (I) purified by vacuum distillation followed by recrystallization from hot ligrom.

In the preparation of ammonia for the reaction with coumalic acid ester (14) an apparatus similar to that described by Schoenheimer and Ratner (15) was used. In a typical run a concentrated water solution of 17.0 gm of NH_4Cl , containing 2.41 atom per cent excess N^{15} , was dropped slowly into 75 cc of boiling 40 per cent NaOH over a period of about 1 hour. The ammonia was carried by a stream of nitrogen into a suspension of 22 gm (0.143 mole) of coumalic ester (I) in 70 cc of H_2O kept cold with ice. Any ammonia not absorbed in the reaction flask was caught in a trap containing HCl. The ester gradually dissolved and the solution turned yellow as hydroxynicotinic acid (II) was formed. The solution of the ammonium salt of hydroxynicotinic acid was allowed to warm up to room temperature and after about 1 hour was cooled with ice and then made alkaline with 70 cc of 40 per cent NaOH. It was found that the yield was decreased and the product was more deeply colored if the NH_4 salt was not cooled before the addition of NaOH. The alkaline solution was boiled for 5 minutes and nitrogen passed through for 30 minutes longer in order to collect all the liberated NH_3 in the HCl trap. The solution was cooled and acidified to Congo red with concentrated HCl, the precipitate was filtered cold and washed with cold H_2O . The tan powder so obtained was purified by solution in dilute $NaHCO_3$ and precipitation with HCl. The yield was 17.7 gm (89 per cent of theory) of hydroxynicotinic acid (II). A series of such runs was made with the NH_4Cl recovered from the trap for each successive run. 99 per cent of the N^{15} used could be accounted for in the hydroxynicotinic acid produced, in the NH_4Cl recovered from the trap of the last run, and in the Kjeldahl digestion of the mother liquors.

Dry powdered hydroxynicotinic acid was decarboxylated as described by von Pechmann and Baltzer (16) and the α -pyridone (III) formed was distilled at atmospheric pressure (b p 285–290°) 141.8 gm of hydroxynicotinic acid so decarboxylated, 20 gm at a time, gave 89.3 gm (92 per cent of theory) of α -pyridone which was purified by recrystallization from ethyl acetate. The yield of recrystallized α -pyridone was 77.3 gm (80 per cent of theory), m p 106–107°.

At this stage heavy hydrogen was introduced into the molecule. α -Pyridone in glacial acetic acid was shaken at 100° with deuterium gas (99.6 atom per cent D) at slightly less than 1 atmosphere of pressure in the presence of platinum catalyst (17) in an apparatus described by Rittenberg and Schoenheimer (18). In a typical run with 26.0 gm of α -pyridone in 25 cc of dry acetic acid with 2.5 gm of platinum catalyst deuterium was consumed as fast as it could be generated. Apparently less catalyst would have sufficed. Theoretical uptake of gas was obtained in about 7 hours. At the end of the reduction the platinum was filtered off, the acetic acid was evaporated, and the α -piperidone (IV) purified by distillation at 134° at 14 mm pressure. Yield, 22.4 gm (83 per cent of theory).

α -Piperidone was converted into *dl*-proline by the method of Heymons (19) with a few modifications (see Fig. 1). The trichloro compound (V) was formed by heating freshly distilled α -piperidone (IV) with 3 equivalents of PCl_5 in a dry xylene solution. The reaction mixture was fractionally distilled at 4 to 5 mm pressure and the portion boiling between 55° and 110° collected. Care had to be taken to keep the solutions dry during the reaction and distillation. The dichloropiperidone (VI) which was formed immediately on the addition of H_2O was further hydrolyzed by boiling with HCl to give the hydrochloride of α, α -dichloro- δ -aminovaleric acid (VII). The solution was concentrated and made just alkaline to litmus with Na_2CO_3 . The α, α -dichloro- δ -aminovaleric acid which precipitated out as colorless crystals was not isolated but was directly reduced to proline (VIII) by the gradual addition of 5 per cent sodium amalgam to the ice-cold, stirred suspension over a period of several hours. The mixture was evaporated to dryness and the proline, isolated by extraction with alcohol, was converted to the copper salt. In the best run 22.4 gm of α -piperidone gave 11.7 gm of copper proline dihydrate (32 per cent of theory).

The combined copper salt from several different preparations was decomposed with H_2S and the *dl*-proline recrystallized from absolute ethyl alcohol.

$\text{C}_5\text{H}_7\text{O}_2\text{N}$ (116.7, corrected for isotope content)

Calculated, N 12.0, found,¹ N 11.9

17.0 atom % D, 2.32 atom % N^{15}

¹All the N analyses reported were done by the Kjeldahl method

The deuterium content is approximately what would be predicted on the assumptions that there are 3 stable deuterium atoms in the synthetic proline, as indicated in Fig. 1, and that the deuterium gas used for the reduction of α -pyridone was diluted by completely exchangeable H from the carboxyl group of the acetic acid solvent and the $=N-H$ group of the α -pyridone.²

Preparation of l(-)-Proline—Proline of the natural optical configuration was obtained from the synthetic *dl*-proline by enzymatic oxidation of the unnatural isomer to α -keto- δ -aminovaleric acid by the method of Krebs (11). It was found convenient to carry out the resolution in 1 gm batches because of the limitations of size of available apparatus and because increasing the concentration of the substrate resulted in less complete oxidation of the *d*(+)-proline. 1 gm of *dl*-proline in a pyrophosphate buffer solution at pH 8.6 was shaken at 39° in an atmosphere of oxygen with a *d* amino acid oxidase solution prepared from dried kidney powder. The total volume of 220 cc was contained in a 500 cc round bottom flask. Oxygen was admitted continually at slightly more than atmospheric pressure and the oxygen uptake roughly measured. After 2 hours nearly the theoretical amount of oxygen was taken up and the process was discontinued after 3 hours. Protein was removed from the resulting solution by means of trichloroacetic acid and the keto acid precipitated as the 2,4-dinitrophenylhydrazone. Yields of the hydrazone varying between 60 and 95 per cent of theory were obtained. After the hydrazone was filtered off, the solution was extracted three times with ether to remove trichloroacetic acid and any excess 2,4-dinitrophenylhydrazine, treated with charcoal, and the colorless filtrate concentrated. Proline was precipitated as the rhodanilate (20) and the free amino acid, still optically impure, was isolated and recrystallized from ethyl alcohol. Variation in yield (78 to 90 per cent) and rotation ($[\alpha]_D = -48^\circ$ to -82°), observed in spite of attempts to keep conditions as nearly constant as possible, were ascribed to variations in the activity of the enzyme preparations used.

The mixtures of *l*- and *dl*-proline obtained from such enzymatic procedures were combined and converted to the picrates. Optically pure *l*-proline picrate was obtained by fractional crystallization, advantage being taken of the greater solubility of *dl*-proline picrate in water, mp 149–150°, $[\alpha]_D^{24} = -20.9^\circ$ (1.5 per cent in water).

² 0.274 mole of α -pyridone requires 1.1 gm atom of H. 0.43 mole of acetic acid was used and the D_2O used for the generation of D_2 was stated to contain 99.6 atom per cent D.

$$\frac{1.1}{1.1 + 0.43 + 0.27} \times 99.6 = 61 \text{ atom \% D in gas phase during reduction}$$

$$\frac{3}{9} \times 61 = 20.3 \text{ atom \% D expected in proline}$$

When 5.56 gm of *l*-proline picrate were decomposed with aniline by the method of Cox and King (21), 1.74 gm (94 per cent of theory) of *l*(-)-proline were obtained

$C_8H_9O_2N$ (116.7, corrected for isotope content)

Calculated, N 12.0, found, N 11.9

$[\alpha]_D^{25} = -82.3^\circ$, 17.8 atom % D, 2.32 atom % N^{15}

In order to determine whether the C-bound deuterium of proline is stable under the conditions of acid hydrolysis, a sample of *dl*-proline containing 17.0 atom per cent D was boiled with 20 per cent HCl for 72 hours. The proline isolated from this solution was found to contain 16.2 atom per cent D. Thus even under these relatively drastic conditions exchange, if there is any, is very slow. This finding is in agreement with the observation that only traces of deuterium were introduced into the molecule when ordinary proline was boiled with HCl and heavy water (22). The biochemical stability of the D on proline was indicated by the unchanged D content after treatment with the kidney powder extract used in the preparation of *l*(-)-proline.

Feeding Experiment—Three adult male rats having a total combined weight of 890 gm were kept on a stock diet consisting of 15 per cent casein, 68 per cent corn-starch, 5 per cent yeast, 4 per cent salt mixture (23), 2 per cent cod liver oil, and 6 per cent of refined cottonseed oil (Wesson oil) for a preliminary period of 7 days during which time the weights of the animals remained constant to within 3 per cent. Isotopic *l*-proline (2.32 atom per cent N^{15} , 17.8 atom per cent deuterium) was then added to the stock diet for the next 3 days. The rats completely consumed 135 gm of diet containing 1.575 gm of isotopic *l*-proline (1.5 mm per rat per day) and maintained constant weight. The urine and feces were collected during the 3 days. At the end of the 3rd day the rats were killed by heart puncture under ether anesthesia and the blood collected in ovalate. The contents of the intestinal tracts were combined with the feces, and the various organs were worked up separately. Small samples of muscle, shaved skin, and bone marrow were taken from each animal for investigation of their proteins (Table III). A sample of the body water distilled from the livers was found to contain 0.011 atom per cent deuterium.

Excreta—The combined urine from the three rats was collected daily and analyzed for total N and isotope. NH_3 was determined and isolated by aeration from K_2CO_3 (24) and urea by isolation as the dioxanthryl derivative (25) of which a recrystallized sample was used for isotope analysis. The combined feces for all 3 days were digested and samples analyzed for total N and N^{15} . The results obtained are shown in Table I.

Blood—The pooled blood, collected in ovalate, was centrifuged, the

plasma pipetted off, and the cells washed with isotonic saline solution and hemolyzed with water. Hemin was isolated from an acetic acid-NaCl solution by the method of Moerner (26). The plasma proteins, precipitated by 6 per cent trichloroacetic acid, were hydrolyzed by boiling for

TABLE I
Partition and Isotopic Distribution of Excretory Nitrogen

The isotope concentration in excess of the natural abundance is expressed as *A*, the analytical value, and *B*, the value calculated on the basis of 100 atom per cent N^{15} in the compound fed

Day of experiment	Source of N	Total N	(A) N^{15} concentration	(B) N^{15} concentration calculated*
		gm	atom per cent	atom per cent
1st	Total urine	0.874	0.061	2.6
	Urea	0.703	0.063	2.7
	NH ₃	0.141	0.061	2.6
2nd	Total urine	0.810	0.073	3.1
	Urea	0.610	0.072	3.1
	NH ₃	0.142	0.074	3.2
3rd	Total urine	0.826	0.076	3.3
	Urea	0.630	0.074	3.2
	NH ₃	0.132	0.091	3.9
	Feces	0.545	0.024	1.0

$$*N^{15} \text{ atom \% calculated} = \frac{N^{15} \text{ atom \% in sample analyzed}}{N^{15} \text{ atom \% in proline fed (i.e. 2.32)}} \times 100$$

TABLE II
Distribution of Isotopic Nitrogen in Blood

	Total N	(A) N^{15} concentration	(B) N^{15} concentration calculated
	gm	atom per cent	atom per cent
Blood whole	1.032	0.016	0.7
Plasma, whole	0.173	0.051	2.2
" non-protein N	0.009	0.053	2.3
" proteins	0.147	0.055	2.4
Erythrocytes	0.729	0.007	0.3
Hemin		0.012	0.5

24 hours with 20 per cent HCl. Samples of whole blood, plasma, hemolyzed cells, hemin, hydrolyzed plasma proteins, and plasma non-protein nitrogen were taken for analysis. The results are shown in Table II.

Carcass—After the removal of the organs and samples of skin, muscle, and bone marrow, the remaining bodies of the three rats were combined,

minced, and extracted several times with 6 per cent trichloroacetic acid to separate the protein from the non-protein nitrogen. The insoluble part was hydrolyzed by refluxing 24 hours in 20 per cent HCl and the humin removed by filtering the chilled solution. Samples of this protein hydrolysate and the non-protein N solution were taken for analysis. The results appear in Table III. The hydrolysate was decolorized with charcoal and used for isolation of "carcass" amino acids.

The samples of bone marrow taken from the femurs of each of the rats were pooled and hydrolyzed directly. The pooled skin samples were extracted with 6 per cent trichloroacetic acid and the insoluble part hydrolyzed with HCl. The muscle samples were treated in the same way as the

TABLE III
Distribution of Total and Isotopic N in Carcass and Organs

		Total N	(A) N ¹⁵ concentration	(B) N ¹⁵ concentration calculated
		gm	atom per cent	atom per cent
Carcass	Non-protein N	4.70	0.010	0.4
	Total proteins	20.31	0.010	0.4
	Muscle "		0.010	0.4
	Skin proteins		0.020	0.9
	Bone marrow		0.052	2.2
Organs	Non-protein N	0.454	0.027	1.2
	Total proteins	1.684	0.035	1.5
	Liver "	0.793	0.041	1.8
	Gastrointestinal tract proteins	0.362	0.029	1.3
	Kidney proteins	0.139	0.030	1.3
	Spleen "	0.083	0.026	1.1
	Lung proteins	0.131	0.025	1.1
	Testes proteins	0.090	0.022	0.9
	Heart "	0.085	0.013	0.6

skin, samples of the bone marrow, skin, and muscle protein hydrolysates were analyzed for N¹⁵ (Table III).

Organs—The livers, washed gastrointestinal tracts, kidneys, spleens, testes, hearts, and lungs were minced, extracted with 6 per cent trichloroacetic acid, and treated for 24 hours with boiling 20 per cent HCl. The trichloroacetic acid extracts were combined and analyzed as non-protein N of the organs. Samples of the protein hydrolysates of the organs were analyzed separately for total N and N¹⁵. The organ hydrolysates were then combined for amino acid isolations and a sample of the combined protein hydrolysates was analyzed for total N and N¹⁵. The results are shown in Table III.

The combined livers after trichloroacetic acid extraction were further

extracted with an alcohol-ether mixture to obtain a sample of liver fat. The fatty acids isolated from this fat contained 0.014 atom per cent deuterium excess.

Glycogen was precipitated from the combined trichloroacetic acid extracts of the organs by the addition of an equal volume of 95 per cent etha-

TABLE IV

Concentration of Isotopes in Amino Acids Isolated after Feeding Isotopic l(-)-Proline

The isotope concentration in excess of the natural abundance is expressed as A, the analytical value, and B, the value calculated on the basis of 100 atom per cent N^{15} and D in the compound fed.

	(A) N^{15} concentration	(B) N^{15} concentration calculated	(A) D concentration	(B) D concentration calculated
	atom per cent	atom per cent	atom per cent	atom per cent
l(-)-Proline fed	2.32	100	17.8*	100
Body water			0.011	0.06
From proteins of carcass				
Cystine	0.008	0.34		
Tyrosine	0.008	0.34	0.002	0.01
Glutamic acid	0.012	0.52	0.014	0.08
Aspartic "	0.009	0.39		
Arginine	0.008	0.34	0.017	0.10
Amidine N	0.008	0.34		
Ornithine	0.008	0.34	0.020†	0.11
α -Amino N	0.004	0.17		
Proline	0.078	3.36	0.600	3.37
Hydroxyproline	0.019	0.82	0.103*	0.58
"Amide N"	0.011	0.47		
From proteins of organs				
Glutamic acid	0.037	1.60	0.046	0.26
Aspartic "	0.028	1.21	0.006	0.03
Arginine	0.022	0.95	0.075	0.42
Amidine N	0.024	1.03		
Ornithine	0.019	0.82	0.088†	0.49
α -Amino N	0.013	0.56		
δ -Amino "	0.015	0.65		
Proline	0.362	15.6	3.09*	17.4
"Amide N"	0.018	0.78		

* These samples were diluted with non-isotopic material prior to combustion for deuterium analyses.

† Calculated from the deuterium concentration of the arginine.

nol (27). The precipitate was centrifuged off, washed with alcohol and ether, thoroughly dried, and analyzed for deuterium. The organ glycogen was found to contain 0.020 atom per cent deuterium.

Amino Acids of "Carcass"—A number of amino acids were isolated from the "carcass" and organ proteins. Their isotopic composition is reported in Table IV.

Cystine and Tyrosine—The decolorized hydrolysates of the carcass protein were concentrated to a small volume *in vacuo* to remove excess HCl and the pH adjusted to 5 to 6 by the addition of $\text{Ba}(\text{OH})_2$. After the solution had stood for several days in the refrigerator, the tyrosine-cystine fraction was filtered off. The cystine was extracted with very dilute NH_4OH at room temperature, crystallized from hot H_2O solution by adjusting the pH to 4.5, and purified by isoelectric precipitation.

Calculated, N 11.7, S 26.7, found, N 11.5, S 26.6

The tyrosine in the undissolved residue was freed of cystine by dissolving it in 5 per cent NaCN and reprecipitating at pH 6, it was then recrystallized from hot water.

Calculated, N 7.7, found, N 7.6

"Amide" Nitrogen—The remaining hydrolysate was made alkaline to phenolphthalein with $\text{Ba}(\text{OH})_2$, the NH_3 was aspirated into dilute H_2SO_4 by nitrogen and analyzed for isotope content.

Dicarboxylic Amino Acids—The barium salts of glutamic and aspartic acids were precipitated by the addition of 5 volumes of alcohol, dissolved in water, and reprecipitated from water with alcohol, the barium was removed and glutamic acid hydrochloride isolated and recrystallized from HCl.

Calculated, N 7.6, found, N 7.5

Aspartic acid was isolated from the mother liquor as the copper salt and recrystallized from hot water.

Calculated, N 7.2, found, N 6.9

Arginine—After the removal of barium from the remaining hydrolysate arginine was precipitated as the flavanate (28), recrystallized, and converted into the monohydrochloride for analysis.

Calculated, N 26.6, found, N 26.6

In order to determine how the isotopic N was distributed among the 4 N atoms of arginine a sample of arginine was converted to the α -toluenesulfonyl derivative (29) and the amidine group split off by boiling with $\text{Ba}(\text{OH})_2$ (30). The NH_3 was collected and α -toluenesulfonyl ornithine isolated and recrystallized from dilute alcohol, m.p. 208–209°.

Calculated, N 9.8, found, N 9.7

Isotope analyses were obtained both on the NH_3 and on the α -toluenesulfonyl ornithine. In a trial it was found possible quantitatively to remove one-half of the N of α -toluenesulfonyl ornithine by the Van Slyke

amino nitrogen method in $\frac{1}{2}$ hour. This was the δ -amino nitrogen, as the α -amino group was blocked. In order to separate the N of the δ - from that of the α -amino group, a solution of 41 mg of α -toluenesulfonyl ornithine in acetic acid was shaken with 10 cc of 30 per cent $\text{Ba}(\text{NO}_3)_2$ for 2 hours. After the removal of barium with a slight excess of sulfuric acid the solution was evaporated *in vacuo* with the repeated addition of $\text{C}_2\text{H}_5\text{OH}$ to remove the nitrous acid. The residue was analyzed by the Kjeldahl method, and the resulting 1.3 mg of α -amino N subjected to isotope analysis.

Proline and Hydroxyproline—After the removal of arginine flavianate the filtrate was freed of flavianic acid by the addition of $\text{Ba}(\text{OH})_2$. The excess barium was removed with sulfuric acid and the solution made strongly acid with HCl and evaporated to a small volume. Proline was precipitated as the rhodanilate (20), regenerated with pyridine, and recrystallized from absolute ethyl alcohol.

Calculated, N 12.2, found, N 12.0

After the removal of proline rhodanilate, hydroxyproline was precipitated as the reneckate (20) which was decomposed with pyridine. The hydroxyproline, which formed colorless crystalline plates after it had stood in the refrigerator for several weeks, was purified by washing with cold absolute methanol, dissolving in a small amount of water, filtering, and reprecipitating with methanol.

Calculated, N 10.7, found, N 10.6, 0.022 atom % N^{15}

Since the hydroxyproline was isolated by means of a reagent which also precipitates proline and the proline obtained from the carcass contained a relatively high concentration of isotope (0.078 atom per cent N^{15}), it was necessary to carry out a "washing out" procedure in order to be sure that the isotope was actually in the hydroxyproline and not all in contaminating proline. To 163 mg of hydroxyproline isolated from the carcass 18 mg of non-isotopic *L*-proline were added to dilute any isotopic proline that contaminated the hydroxyproline from the animals. The mixture was dissolved in a few drops of water and hydroxyproline was precipitated from the mixture by means of methanol. The first crop, 141 mg, was used for the analyses.

Calculated, N 10.7, found, N 10.7, 0.019 atom % N^{15}

Three more crops of hydroxyproline, having a total weight of 19 mg, were removed by evaporating the solution to a small volume and adding methanol. After a total of 160 mg had been removed, proline was precipitated from an ethanol solution of the evaporated mother liquor by the addition of a saturated solution of CdCl_2 in 95 per cent ethanol (31). 29 mg of CdCl_2 proline double salt were obtained.

Calculated, N 4.7, found, N 4.7, 0.018 atom % N^{15}

From these figures it can be calculated that the original sample of hydroxyproline isolated from the carcass had been contaminated by 3 to 5 per cent proline. Since the proline present as a contaminant after the washing out had the same N^{15} concentration as the hydroxyproline, the value of 0.019 atom per cent N^{15} may be taken as the true isotope concentration of the carcass hydroxyproline.

Amino Acids of Organs—The following amino acids were isolated from the combined organ protein hydrolysates by the methods used for the carcass: glutamic acid hydrochloride (calculated, N 7.6, found, N 7.6), copper aspartate (calculated, N 7.2, found, N 6.9), arginine hydrochloride (calculated, N 26.6, found, N 26.3), proline (calculated, N 12.2, found, N 12.0). The isotope analyses of these compounds are found in Table IV.

Arginine—In order to study the distribution of N^{15} among the N atoms of the organ arginine a 20 mg sample of arginine hydrochloride was refluxed with a solution of $Ba(OH)_2$ for 30 hours and the NH_3 liberated from the amidine group was swept out by means of a stream of N_2 gas into H_2SO_4 . 2.44 mg of N were obtained from the amidine group (2.66 mg, theory). Barium was removed from the remaining ornithine solution which was found to contain 2.61 mg of N (2.66 mg, theory) and the two samples were analyzed for N^{15} (Table IV).

The silver oxide oxidation method of Herbst and Clarke (32) was used to obtain separate samples of N from the α - and δ - NH_2 groups of the organ ornithine. These authors found that β -aminopropionic acid was not oxidized by Ag_2O under conditions which brought about oxidation of α -amino acids, and concluded that only the α -amino groups responded to this treatment.

In a trial run with authentic ornithine dihydrochloride (≈ 5 mg of N) the N was divided into two approximately equal fractions by this method. The NH_3 fraction (≈ 2.58 mg of N) was isolated from the volatile portion by the method of Weber and Wilson for separating NH_3 from amines (33), and presumably came from the α -amino group. From the residues 1.85 mg of non-ammoniacal N was recovered and was assumed to have arisen from the δ -amino group of the ornithine.

A sample (approximately 8 mg of N) of arginine monohydrochloride isolated from the organs was boiled with $Ba(OH)_2$ solution for several days until 98 per cent of the theoretical amount of N in the amidine group was recovered as NH_3 . After excess barium was removed as the carbonate, the ornithine in solution was oxidized with Ag_2O . 1.07 mg of NH_3 -N and 1.11 mg of residue N were obtained. The N^{15} analyses of these fractions are reported in Table IV as α -amino N and δ -amino N respectively.

DISCUSSION

Absorption from Gastrointestinal Tract—Less than 3 per cent of the isotopic nitrogen fed was recovered in the feces (Table V), at least 97 per cent

of the proline of the diet must have been absorbed across the wall of the gastrointestinal tract

Urine—Nearly 40 per cent of the N^{15} fed as proline was recovered in the urine. The concentration of isotope was of the same order of magnitude in the urea as in the NH_3 isolated from the urine. However, by far the greater quantity of N^{15} was present in urea, as the urine contained about 5 times as much urea as NH_3 nitrogen (Table I). As almost all of the isotope in the urine could be accounted for in the urea and the NH_3 , no other compound rich in isotope can have been present in large amount.

Distribution of Isotopic Nitrogen in Carcass and Organs—The nitrogen of the proline that was absorbed was found to be distributed throughout the body in the protein and non-protein nitrogen fractions of the various tissues and organs (Table III). The anatomical distribution of the nitrogen of the secondary amino acid, *l*(-)-proline, was in a general way similar to

TABLE V

Balance of Nitrogen Isotope after Feeding Isotopic l(-)-Proline

The values are calculated from the total nitrogen of the fractions and their isotope concentrations

	Fraction of administered N^{15} recovered
	per cent
Feces	2.6
Urine	39.7
Non-protein nitrogen	12.7
Protein nitrogen	59.8
Total isotope recovered	114.8

that found after the feeding of the primary amino acid, *l*(-)-leucine (34). The livers, gastrointestinal tracts, and kidneys contained a higher concentration of isotopic nitrogen than did the other organs investigated, an indication of the more rapid uptake of proline in these organs. The concentration of isotope found in the proteins of any organ after the administration of an isotopic amino acid will depend not only upon the rate of protein turnover but also upon the quantity of that amino acid normally present in the proteins of the organ under investigation. The concentration of isotopic nitrogen in the proteins of the skin is higher than would have been expected on the basis of experiments with other isotopic amino acids (34, 35). This may be due in part to the high concentration of proline in connective tissue, as is evidenced by its high concentration in gelatin (36) and elastin (37).

The bone marrow contained a very high concentration of N^{15} . There are no available figures from other isotopic amino acid feedings with which

to compare this finding. It is at least in part a reflection of the rapid cellular proliferation of bone marrow.

Blood and Hemin—The proteins of the plasma were found to be richer in isotope than those of any other tissue analyzed, as was also the case when isotopic glycine, *l*(-)-leucine, and *d*(+)-leucine were fed (38). The erythrocytes had a low concentration of isotope.

The nature of the starting materials from which animals synthesize the pyrrole rings of hematoporphyrins has long been a subject of speculation. The possibility that the amino acids proline, hydroxyproline, glutamic acid, and pyrrolidonecarboxylic acid are involved in such a synthesis has, on the basis of their structural similarity to pyrrole, been suggested by Abderhalden (39) and by Lusk (40). With the object of shedding some light on this question, hemin was isolated from the blood of the animals fed isotopic *l*(-)-proline. However, in view of the slow rate of synthesis of hemoglobin, the short duration of the feeding experiment, and the low isotope concentration of the *l*(-)-proline fed, no clear conclusions can be drawn as to the rôle of proline in porphyrin synthesis. The hemin isolated after *l*(-)-leucine had been fed contained a lower percentage (0.14) of the labeled nitrogen than did the red blood cells (0.29), whereas after *l*(-)-proline had been fed for the same length of time the hemin contained a higher percentage (0.52) than the whole red cells (0.30). About the same concentration of N^{15} was found after a 3 day administration of isotopic *l*(-)-proline as after 9 days of a diet containing isotopic ammonia (41). These results leave open the possibility that the pyrrole nucleus of porphyrins can be derived intact from proline.

Replacement of Tissue Proline by Dietary Proline—If the proline content of casein is taken as 8 per cent (42), the 135 gm. of diet which contained 15 per cent of casein had an approximate total of 1.6 gm. of non-isotopic proline. If the small amount of proline contributed by the 5 per cent of yeast be neglected, it can be estimated that the rats consumed approximately 1.6 gm. of non-isotopic proline in addition to the 1.575 gm. of isotopic proline (2.32 atom per cent N^{15} excess) during the 3 day experiment. The average N^{15} content of the proline of the diet was therefore $2.32 \times 1.6/3.2 = 1.2$ atom per cent excess. Since the proline isolated from the carcass contained 0.078 atom per cent N^{15} , at least $(0.078 \times 100)/1.2 = 7$ per cent of all the proline present in the carcass protein was replaced by dietary proline in 3 days. It can similarly be computed that at least 30 per cent of the proline of the organs was replaced by proline from the diet in 3 days. Since the total amount of proline in the diet is well within the normal range and the weights of the animals remained approximately constant throughout the feeding experiment, these figures may be taken as a measure of the minimum rates of normal replacement of tissue proline by dietary proline.

The use of both D and N¹⁵ as markers of the same amino acid molecule makes it possible to follow the metabolic fate of the carbon chain and of the amino group separately (34). After the administration of doubly marked *l*(-)-leucine, the leucine isolated from the proteins of the rats contained both isotopes, but in greatly altered ratio, indicating that leucine was rapidly deaminated and aminated (34). When isotopic *l*(+)-lysine was fed, the ratio of the isotope concentrations of the lysine isolated was the same as in the compound fed, showing that none of the N of the α -NH₂ group had been replaced by nitrogen from other sources (43). In the present case also both isotopes were found in the proline isolated. The isotope ratio (N¹⁵/D) in the proline fed was $2.32/17.8 = 0.13$, in the proline isolated from the carcass, $0.078/0.600 = 0.13$, and in the proline isolated from the organs, $0.362/3.09 = 0.12$. Analytically these findings resemble the results with lysine rather than with leucine. The interpretation, however, is somewhat different. Unlike both lysine and leucine, proline is dispensable and can be synthesized from ornithine (12) and probably from other metabolites. Since the secondary amino acid proline cannot lose N¹⁵ by N transfer reactions without prior ring opening, a change of isotope ratio (N¹⁵/D) could occur only as a result of extensive proline synthesis from compounds richer in one isotope than in the other. The actual finding of a ratio of isotopes approximately the same in the proline isolated as in that fed indicates that the proline synthesized was formed either from compounds of such low isotope content as to have little effect on the ratio or from metabolic derivatives of proline with roughly the same or compensating ratios of N¹⁵/D.

Conversion of Proline to Hydroxyproline—The hydroxyproline isolated from the protein of the carcass contained a relatively high concentration of both N¹⁵ and D (Table IV), proving that part of the proline was converted to hydroxyproline *in vivo*. Except for proline, the hydroxyproline was richer in isotope than was any other carcass amino acid isolated. From a comparison of the respective isotope ratios (N¹⁵/D) of the hydroxyproline (0.18) and the proline (0.13) it appears that the conversion of proline to hydroxyproline occurred with the loss or labilization of less than one-half of the carbon-bound hydrogen. If it is assumed that the hydroxyproline was formed from proline which had the same isotope content as that isolated from the carcass, the N¹⁵ figures would indicate that about one-quarter of the carcass hydroxyproline had been so produced during the 3 days of the feeding experiment.

Conversion of Proline to Glutamic Acid—Glutamic acid is known to derive its N from many sources (34, 44, 41, 45) and the finding of N¹⁵ in glutamic acid after administration of the isotopic proline would not alone furnish conclusive proof of the conversion of the carbon skeleton of proline into

glutamic acid The finding that glutamic acid, in spite of its reactivity and abundance in tissue proteins, contained appreciable amounts of D proves that the carbon skeleton of part of the glutamic acid was in fact derived from proline—a reaction previously demonstrated for tissue slices (5, 6) Of the 6 H atoms labeled with D in the proline fed, only 2 remain stable in the glutamic acid formed, those on the β -carbon atom (46) The extent of conversion is therefore greater than is indicated by a direct comparison of the D concentration of the organ glutamic acid with that of the organ proline

Transamination in the usual sense (47) cannot occur with the secondary amino N of proline However, once the proline ring has oxidatively opened, the N of the resulting glutamic acid can enter into nitrogen transfer reactions The finding of small but significant amounts of N¹⁵ in cystine, tyrosine, and aspartic acid can be thus explained

Conversion of Proline into Arginine—That the carbon skeleton of proline is in part converted into arginine *in vivo* is shown by the significant concentration of D found in the arginine isolated from the organ proteins It has previously been shown that when deuterio ornithine was fed to mice the proline isolated from the proteins of their bodies contained appreciable amounts of deuterium (12) These two findings considered together show that proline and ornithine are mutually interconvertible in the animal The fact that proline can serve as a precursor of arginine explains the definite though limited ability of the rat to synthesize arginine (48)

The organ arginine isolated contained, in addition to D, significant amounts of N¹⁵ A portion of the N¹⁵ was found in the amidine group, as was the case when isotopic NH₃ (41), tyrosine (44), and *l*(-)-leucine (34) were fed, and was presumably introduced during the process of urea synthesis Significant amounts of the N¹⁵ of the proline fed were found in both the α - and δ -amino groups of the ornithine derived from the arginine If the concentration of deuterium in the ornithine is taken as a measure of the quantity of ornithine derived from proline, the expected concentration of N¹⁵ in that nitrogen atom arising directly from proline would be about 0.014 atom per cent As the isotopic values for the N in both the α and δ positions of the ornithine are of this order of magnitude, they give no clue as to the side of the ring N on which the cleavage actually occurs

The reasons for the selection of the pathway from proline to ornithine indicated in Fig. 2 are first, that amination in the α position is a well recognized biological process, whereas introduction of an amino group in the δ position is not, and secondly, the compound (c), α -keto- δ -aminovaleric acid, has been shown by Krebs to be the product of the action of *d*-amino acid oxidase of kidney on both *d*(+)-proline and *d*(-)-ornithine The isotope found in the α -nitrogen of ornithine has, in this scheme, an origin similar to that in the α position of aspartic acid, tyrosine, and cystine

Metabolic Relationships of Proline, Hydroxyproline, Ornithine, and Glutamic Acid—In considering the relationships between the 5-carbon amino acids, the following observations must be taken into account (1) When proline containing N^{15} and D was fed, both isotopes were found in the ornithine, glutamic acid, and hydroxyproline isolated from the animals N^{15} was found in the δ - as well as the α -amino group of ornithine (2) When

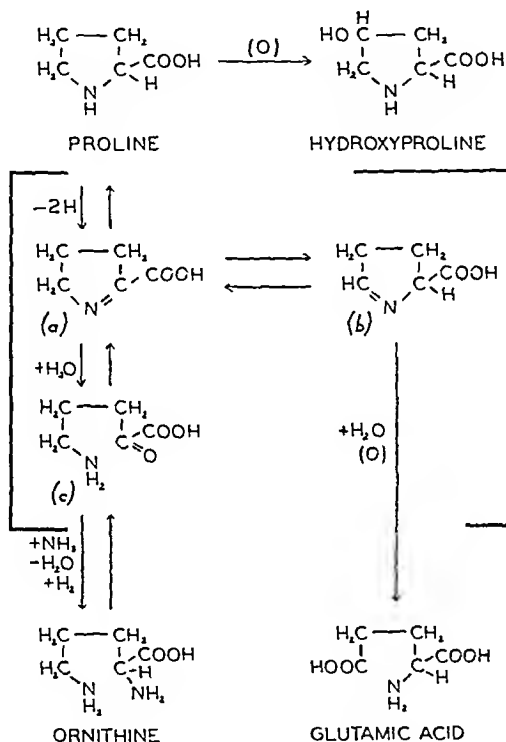


FIG 2 Metabolic interrelationships of proline, hydroxyproline, glutamic acid, and ornithine. The over-all conversions indicated have been found to occur in the intact animal. There is as yet no convincing evidence for the *in vivo* conversion of hydroxyproline and glutamic acid into proline. Hypothetical intermediates are enclosed in brackets.

deuterio ornithine was fed, proline and glutamic acid isolated contained D (12) (3) Pyrrolidonecarboxylic acid (5, 6) and δ -hydroxy- α -amino-valeric acid (6) have on other grounds been eliminated as probable intermediates in the conversion of proline to glutamic acid. The scheme of possible intermediates in the interconversions proposed in Fig 2 accounts for the experimental findings. If the first step in the oxidation of proline

were a dehydrogenation similar to that generally held to occur in the case of the primary amino acids (49), a pyrrolinecarboxylic acid, which could exist in tautomeric forms, would be the product. Hydrolysis and oxidation of (b) would result in the formation of glutamic acid, hydrolysis of (a) would result in the production of α -keto- δ -aminovaleric acid (c) which could undergo reductive amination to ornithine. The δ -amino group would thus be derived directly from proline and the α -amino group from the body pool of nitrogen. The formation of the intermediates (a), (b), (c) would also give a plausible route for the conversion of ornithine into glutamic acid and proline.

SUMMARY

1 Proline was so synthesized that the carbon skeleton was marked by stably bound deuterium and the amino group by N^{15} . The optically natural component, *l*(-)-proline, was prepared from the racemic compound by the use of *d*-amino acid oxidase.

2 Isotopic *l*(-)-proline was added to the normal stock diet of three adult rats for 3 days and was well absorbed. Nearly 40 per cent of the isotopic N was recovered in the urinary urea and NH_3 . The remainder was distributed in the protein and non-protein nitrogen fractions of the organs and tissues. The highest concentration of N^{15} was found in the plasma proteins and bone marrow, the next highest in the proteins of the liver and the other internal organs, while the concentration in the muscle was relatively low.

3 The isotope concentration of the proline isolated indicated that at least 30 per cent of the proline of the proteins of the internal organs and 7 per cent of the proline of the "carcass" proteins was replaced by dietary proline in 3 days. The ratio of isotopes (N^{15} D) was approximately the same in the proline isolated as in that fed.

4 The *in vivo* oxidation of proline to glutamic acid was shown by the finding of deuterium as well as N^{15} in the glutamic acid isolated.

5 The finding of deuterium in the ornithine isolated proves that a portion of the ornithine was derived from proline. N^{15} was found not only in the amidine group of arginine isolated but also in both the α - and δ -amino groups of the ornithine prepared from this arginine.

6 From the isotope concentration of the hydroxyproline isolated from the proteins of the carcass it was estimated that about one-quarter of this amino acid had arisen by the oxidation of proline in 3 days.

7 Small but significant amounts of isotopic nitrogen were found in other amino acids isolated from the proteins.

8 A scheme of possible reactions in the biological interconversions of proline, hydroxyproline, ornithine, and glutamic acid is presented.

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A MICROTITRATION METHOD FOR THE DETERMINATION OF SMALL AMOUNTS OF CITRIC ACID

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When citric acid is oxidized at room temperature with potassium permanganate in the presence of potassium bromide, pentabromoacetone is formed. According to the colorimetric method of Pucher, Sherman, and Vickery (1) to determine citric acid, the pentabromoacetone is extracted with petroleum ether and treated with sodium sulfide, whereby a yellow color, proportional in intensity to the citric acid originally present, is produced in the aqueous phase. Experience with this method has brought certain disadvantages to light. It has been found that readings must invariably be made without delay and, in addition, occasional lots of petroleum ether have been encountered which do not give the same ratio between the extinction coefficient observed and the quantity of citric acid taken as was found with the solvent used in the original investigation. Accordingly a calibration curve must be constructed for each lot of ether. Attention was drawn to this point in the earlier paper.

Recently it has not been possible to obtain petroleum ether similar to that previously employed. Data are shown in Table I that illustrate the difference in the ratio for the petroleum ether previously used and that now obtainable. Nevertheless volumetric estimation of the bromine showed that the yields of pentabromoacetone formed by the oxidation of the citric acid were, in both sets of experiments, in accord with the previous observations of Pucher, Vickery, and Leavenworth (2).

In order to eliminate the uncertainties in the colorimetric method, a microtitration procedure, based upon experimental evidence previously described (2-4), has been developed as a substitute. It has been found possible to estimate as little as 0.05 mg. of citric acid with an accuracy of ± 5 per cent, and the new procedure may be applied equally as well as the old to the analysis of trichloroacetic acid filtrates obtained from animal tissues and to the organic acid fractions prepared from plant tissues.

Reagents—

Sulfuric acid, 18 N. A mixture of equal volumes of concentrated acid and water.

Potassium permanganate, 1.5 N. 47.4 gm. in 1000 ml. of solution.

Potassium bromide, 1.0 M. 11.9 gm. in 100 ml. of solution.

Sodium sulfide, 4 per cent 4 gm of crystalline sodium sulfide in 100 ml of solution, prepared fresh every 2 to 3 days

Petroleum ether B p 35-50°

Hydrogen peroxide Commercial 3 per cent solution

Hydrogen peroxide (halogen-free) 16 to 18 ml of 18 N sulfuric acid are poured slowly into 100 ml of water, the mixture is cooled to 5°, and about 10 gm of fresh solid sodium peroxide are added slowly so that the temperature does not rise above 30°. The final solution, which should be acid to Congo red paper, is cooled to 5° and the supernatant fluid is decanted or centrifuged from the precipitated sodium sulfate. The solution contains from 2.5 to 3.5 per cent of hydrogen peroxide.

TABLE I
Recovery of Citric Acid by Colorimetric Method and by Titration Method

Citric acid present	Citric acid found		
	Colorimetric method		Titration method
	Old ratio*	New ratio†	
mg	mg	mg	mg
0.128	0.095	0.130	0.124
0.128	0.101	0.139	0.130
0.128	0.097	0.132	0.128

* Mg of citric acid = $E \times 0.920$, from calibration curve obtained with earlier lots of petroleum ether

† Mg of citric acid = $E \times 1.26$, from calibration curve obtained with recent lots of petroleum ether

Silver nitrate, 0.01302 N 2.2116 gm of pure silver nitrate in 1000 ml of solution 1 ml is equivalent to 0.500 mg of citric acid or to 1.866 mg of silver chloride. The exact normality is checked by a gravimetric chloride determination in 100 ml aliquots.

Ammonium thiocyanate, 0.01302 N 0.99 gm in 1000 ml of solution

Ferric ammonium sulfate 30 gm dissolved with 100 ml of water

Sodium sulfate 20 per cent solution of a halogen-free reagent

Method

Filtrates suitable for the determination of the citric acid content of biological material may be prepared according to the directions of Pucher, Sherman, and Vickery (1), suitable aliquots are transferred to 100 ml beakers. To each aliquot 3 ml of 18 N sulfuric acid are added and the solution is boiled for 5 minutes or until a volume of about 35 ml is attained.

The solutions are cooled to 20–25° and the citric acid is oxidized by the addition of 2 ml of 1 M potassium bromide and 5 ml of 1.5 N potassium permanganate. The solution is allowed to stand at room temperature for 10 minutes and is then cooled in an ice bath to 10° or lower and decolorized by the dropwise addition of 3 per cent hydrogen peroxide.

The oxidation mixture is transferred to a short stem (2 to 2.5 cm), 125 ml pear-shaped separatory funnel previously cleaned with chromic-sulfuric acid, each beaker being carefully rinsed into the corresponding funnel with 25 ml of petroleum ether used in small portions. The funnel is shaken vigorously for about 30 seconds and the aqueous layer is drawn off into the original beaker. The ether is transferred to a second short stem separatory funnel. The aqueous solution is shaken again with 20 ml of petroleum ether and then discarded. The second ether extract is added to the first and, after a settling period of at least 1 minute, the small aqueous layer is drawn off and the end of the funnel is wiped dry. The ether extract is washed four times with 3 to 4 ml quantities of water to remove traces of inorganic halide. A settling period of at least 1 minute is allowed after each washing, and the ends of the separatory funnels must be wiped dry after each operation. The pentabromoacetone is decomposed by shaking the petroleum ether solution successively with 3 ml and with 1 ml portions of 4 per cent sodium sulfide solution, and then with 2 ml portions of water until no further color is observed in the wash solution. One washing is usually sufficient. The extracts are successively drained into a 25 ml Erlenmeyer flask and, when extraction is complete, 0.5 ml of 6.0 N sulfuric acid is added and the solution is boiled for 1 to 2 minutes to expel hydrogen sulfide. The addition of small angular quartz boiling stones greatly facilitates this step. The solution is cooled to 15° or below and, when convenient, 0.5 ml of 1.5 N potassium permanganate is added in order to oxidize substances which interfere with the subsequent titration of the bromide. The solution is stirred for about 10 seconds and decolorized by the rapid addition of halogen-free hydrogen peroxide, 1 ml of concentrated nitric acid, 1 ml of 30 per cent ferric ammonium sulfate, 1 ml of 20 per cent sodium sulfate, and, from an accurate pipette, exactly 2 ml of the standard silver nitrate solution are then successively added. After 5 minutes, the solution is titrated with the ammonium thiocyanate solution delivered from a micro burette. As the end-point is approached, the flasks are shaken vigorously for 10 to 20 seconds and the titration is then completed to the first salmon-pink color. The maximum difference between duplicate determinations should not be greater than 0.02 ml.

The result is calculated from the equation

$$\text{Mg citric acid} = 1.166 [0.500 (\text{ml AgNO}_3 \text{ minus ml NH}_4\text{NCS}) - 0.020]$$

DISCUSSION

The success of the procedure depends upon careful attention to details of technique. Thorough washing to insure the removal of traces of inor-

TABLE II
Recovery of Citric Acid by Titration Method

Citric acid present	Citric acid found*	Actual recovery	Corrected recovery†
mg	mg	per cent	per cent
0 051	0 042	86 27	100 6
0 128	0 110	85 93	100 2
0 256	0 220	85 93	100 2
1 28	1 077	84 16	98 1
1 92	1 622	84 48	98 5
3 20	2 739	85 61	99 8

* Corrected for reagent blank

† Corrected by the empirical factor 1 166

TABLE III
Citric Acid Content of Dry, Fat-Free Chicken Tibias by Colorimetric and Titration Method, Duplicate Analyses by Titration Method

Sample No *	Colorimetric method†	Titration method‡
	per cent	per cent
335	0 530	0 472
369	0 700	0 688
466	0 746	0 780
473	0 726	0 720
474	0 716	0 688
495	0 726	0 780
642	0 600	0 626
675	0 500	0 502
676	0 650	0 628
947	0 416	0 414
811		1 00
		1 01
812		1 07
		1 05
815		0 720
		0 740
821		0 704
		0 694

* 50 mg of each sample were taken for analysis

† These values were obtained before the change in the calibration curve had been encountered

‡ The same samples subsequently analyzed by the titration method

ganic halides from the petroleum ether solution of the pentabromoacetone is of the utmost importance. The range of the method is from 0.05 to 1.0 mg of citric acid. If more than the larger amount is present in the aliquot taken, it is only necessary to add more standard silver nitrate solution, either before or even after the addition of the first drop of ammonium thiocyanate solution, and then to continue the titration to the end-point.

The equation for the calculation of the citric acid values includes two experimentally determined empirical quantities. A blank determination is carried out in which water is used instead of a citric acid solution. The blank requires from 0.03 to 0.05 ml of 0.01302 N silver nitrate and is equivalent on the average to 0.020 ± 0.005 mg of citric acid, which quantity is accordingly subtracted. In addition, as was pointed out by Pucher, Vickery, and Leavenworth (2), the theoretical citric acid equivalent of the standard silver nitrate used must be multiplied by an empirical factor to obtain the actual citric acid content of the samples. This factor, calculated from the results of twenty-five recent analyses of samples which contained from 0.051 to 3.20 mg of citric acid, was found to be 1.166 ± 0.023 . This figure agrees moderately well with the factor of 1.13 found when the original method was developed (1).

In Table II are recorded representative data which show recoveries of known amounts of citric acid. Table III shows a comparison of results by the titration method with the earlier colorimetric method, as applied to the determination of citric acid in chicken tibias, as well as a series of duplicate determinations to illustrate the precision that can be obtained. These data indicate that satisfactory values for citric acid can be obtained over a wide range by the microtitration technique and that the results agree well with those obtained by the earlier colorimetric procedure.

SUMMARY

A microtitration method has been developed for the estimation of small amounts of citric acid, which is recommended as a substitute for the earlier colorimetric method of Pucher, Sherman, and Vickery (1). The new procedure is independent of those factors which necessitate a frequent checking of the calibration curve used in the colorimetric procedure.

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A RAPID METHOD FOR ESTIMATING SERUM PROTEINS

FORMULA FOR CALCULATING SERUM PROTEIN CONCENTRATION FROM THE REFRACTIVE INDEX OF SERUM

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(Received for publication, December 13, 1943)

The importance of having available a rapid method for estimating the concentration of serum proteins in emergency situations has long been recognized. Such information would help in selecting patients that require replacement therapy and in determining the type of replacement therapy required. Rapid methods for measuring the hemoglobin concentration in blood have been adequate, however, speedy methods for measuring the serum proteins, such as the falling drop method, have not proved to be entirely adapted to our needs. Estimation of protein concentration from the refractive index of the serum is rapid, easy to perform, and economical of material, requiring only 1 drop of serum, no reagents are required but the use of a refractometer is necessary.

The principle of measuring serum proteins refractometrically is an old one, having been introduced by Strubell in 1900 (1) and systematically studied by Reiss (2), Robertson (3), Schorer (4), and others. References to the early studies in this field are included in an article published by Reiss in 1913 (5). Reiss' method in brief consisted in measuring the difference between the refractivity of serum and water at 17.5°, subtracting a constant for the non-protein components, and dividing the remainder by the change in refractivity produced by a 1 per cent protein solution¹. Later, Robertson (6) reported different protein and non-protein constants than those obtained by Reiss and showed that the refractive index of serum albumin is less than that of serum globulin. Schorer (4), Robertson, and others concluded that the method of estimating serum protein by Reiss' formula yielded erroneous results in pathological sera. This led to the elaboration of a more time-consuming method by which Robertson (7) estimated the albumin, globulin, and non-protein constituents separately.

¹ Reiss' formula may be expressed as follows

$$\text{Pr} = \frac{R_s - R_w - 0.0027}{0.00172}$$

Pr = serum protein, gm per 100 ml, R_s = refractive index of serum, R_w = refractive index of water, 0.0027 = arbitrary correction for salts and non-electrolytes in serum, 0.00172 = Reiss' figure for the refractive index of a 1 per cent protein solution

The refractometric method for estimating serum proteins in the past had some clinical usage until it was shown by various investigators, including Linder, Lundsgaard, and Van Slyke (8) and Guillaumin, Wahl, and Laurencin (9), that the values obtained by the refractometer were higher than those obtained by standard chemical methods. As a consequence, the refractometric method has not been employed to any great extent in recent years.

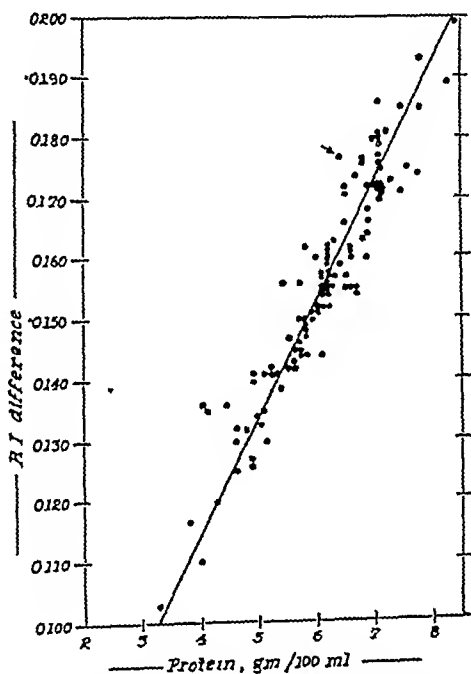


FIG 1 Refractometer values plotted against serum protein. The equation for the statistically calculated regression line is $Y = 0.0181X + 0.00181$. The point indicated by the arrow represents a specimen of high bilirubin concentration.

The measurements of Linder, Lundsgaard, and Van Slyke were made on plasma and, although not stated, presumably on serum. Refractometric values by the Kjeldahl method were found to be in normal plasma (10). On the other hand, Neuhausen and Riich (11) found their values and those of Austin and Camac

to be higher than those of Van Slyke. The equation for the regression line was used in the results obtained per 100 ml of plasma. The constants in the equation are ably good. The Kjeldahl method is a standard method for the estimation of protein.

between the proteins estimated refractometrically and the dry weight as well as the specific gravity of the serum

Since refractometric measurements offer the advantages of speed and simplicity, it occurred to us that the correlation between the refractivity and protein concentration of serum might be reinvestigated. Accordingly, simultaneous measurements were made of the refractivities and protein concentrations of sera obtained from 112 patients suffering from miscellaneous pathological conditions. The refractivity measurements were made by means of an Abbe refractometer, the protein measurements were made by the Kingsley method (13) checked daily with serum analyzed by the Pregl method (14) for nitrogen. The results of these measurements are plotted and shown in Fig. 1.

Method

The refractivity measurements are made by placing 1 drop of serum in the chamber of an Abbe refractometer and reading the refractive index. From this value is subtracted the refractive index of water at the same temperature. The values of the refractive index of water may either be measured directly or may be obtained for any given temperature from prepared refractivity tables (15). For purposes of this measurement it is sufficiently accurate to calculate the refractive index of water at room temperature by the following formula

$$R\ I\ \overset{(t)}{H_2O} = 1.3332 - [0.0001(t) - 0.0018]$$

If distilled water is not available for direct measurement, tap water may be employed without introduction of any appreciable error.

When the differences in the refractive index of serum and water are plotted against the serum protein concentrations, it will be seen (Fig. 1) that an excellent linear correlation is obtained. The statistically calculated regression line derived from this correlation may be expressed by the equation

$$Pr = 510\ R\ I_{diff} - 1.81$$

(Pr = gm of protein per 100 ml, $R\ I_{diff}$ = the refractive index of serum minus the refractive index of water). The standard deviation for this regression line is equal to 0.31 gm per 100 ml.

DISCUSSION

Greatly increased amounts of bilirubin or fats in serum might possibly alter the refractometric values in relation to concentrations of protein. No systematic study has been made of such possible deviations. In sev-

eral of the specimens in our series the concentrations of non-electrolytes such as cholesterol, urea, and sugar were elevated and one specimen shown on Fig 1 contained a high concentration of bilirubin. In these specimens the agreement of the calculated with the found values was not significantly affected. This method is recommended especially for use in traumatic shock, and in such cases large amounts of fats or bile pigments will seldom be found.

As a method for quick clinical guidance we find from our data that protein values less than 5 gm per 100 ml are rare unless the refractive differences at room temperature are less than 0.0141, protein values greater than 7 gm per 100 ml are rare unless the differences are greater than 0.0170².

SUMMARY

A formula is presented for calculating the concentration of serum protein from refractivity measurements. The method is economical in time, easy of manipulation, requires no reagents, and is useful in guiding therapy in cases of shock.

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² Most of these analytical measurements were made by Miss Margaret Scully

GLUTATHIONE AS AN ESSENTIAL GROWTH FACTOR FOR CERTAIN STRAINS OF NEISSERIA GONORRHOEAE*

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Although the gonococcus has often been described as being "fastidious" in its nutritional requirements, little has been reported on specific growth factors. Lankford and Snell (1) have recently found that 10 to 15 per cent of their strains require glutamine for primary isolation. Such strains, they state, readily produce "normal mutants" on subculture, which no longer require glutamine.

Several reports have appeared on the effect of cystine, some workers (2, 3) stating that it was inhibitory and others (4) that it stimulated growth. Boon found that stock strains required cystine when grown on an enzymatically digested casein medium and that optimum growth was obtained at a cystine concentration of between 0.025 and 0.075 per cent. He regards this as only a type requirement, since certain other sulfur compounds including barium sulfide and even sulfur itself were capable of replacing cystine. Because of the high optimum level he suggests that the function of cystine is "evidently something more than as a 'building stone'." McLeod had earlier observed that cystine was inhibitory in high concentrations but was sometimes stimulatory in low concentrations. His experiments emphasized the differences between strains and also the importance of other constituents of the medium, particularly the colloids, as determining whether any amino acid would stimulate or inhibit growth.

The protein-free medium of Mueller and Hinton (5) has been used as the basis for an investigation of the growth factors for the gonococcus. This medium, which gives excellent growth of both freshly isolated and stock strains, consists of meat infusion, starch, acid hydrolysate of casein, and agar. It has been found¹ that freshly isolated strains will grow if the meat infusion is replaced by a mixture of glucose, sodium and potassium phosphates, and a magnesium salt, although the growth is not so vigorous.

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¹ Gould, R. G., Kane, L. W., and Mueller, J. H., *J. Bact.*, in press.

The meat infusion thus contains a factor (or a group of factors) which stimulates the growth of these strains but none that is essential

However, it has been observed that certain stock strains failed entirely to grow in the absence of meat infusion and later several strains, which grew without meat infusion when freshly isolated, were observed to change after some weeks of daily subculturing on the Mueller-Hinton medium to variants which were subsequently completely dependent on the presence of meat infusion

An investigation of the nature of the factor necessary for the growth of these exacting strains is the subject of the present communication

Method of Assay

Cultures were transferred daily on slants of the Mueller-Hinton medium and the experimental plates were streaked in such a way that well isolated colonies were formed. After 40 hours incubation in a candle jar the diameters of five or more typical, well isolated colonies were measured by means of a low power microscope with a micrometer eyepiece. The size of an individual colony was found to be strictly proportional to the time of incubation during the period between 15 and 40 hours of incubation.

The variation in the size of such colonies on any one plate was found to be less than ± 10 per cent, and the average values obtained for the same strain on the same medium in different experiments were ordinarily reproducible within ± 10 per cent. Different strains varied considerably in the colony size, but any one strain was found to give essentially constant results over long periods of time. The main sources of error in this method of assay, exclusive of the sudden change in growth requirements mentioned above, were a "crowding" effect due to large colonies or too heavy inoculation, and incomplete mixing of the medium.

The basal medium consisted of the following constituents

Casamino acids (Difco)*	1.50 gm	Na HPO ₄	0.28 gm
Starch	0.15 "	MgSO ₄ 7H ₂ O	0.045 "
Glucose	0.15 "	Agar	1.7 "
KH ₂ PO ₄	0.10 "	Water to make	100 cc

* This amount supplied about 0.7 gm of amino acids and 0.6 gm of NaCl

The pH was adjusted to 7.4 to 7.6 and the medium autoclaved for 5 minutes at 10 pounds in 20 cc amounts. One loopful of a 24 hour starch-agar culture was suspended in 0.5 cc of saline and the plates were streaked with one loopful of this suspension. Most of the inoculum was spread over a small area at the top of the plate, some confluent growth was always obtained on this area except in the presence of strong inhibitory agents. The criterion of growth was the development of isolated colonies on the rest of the plate, particularly on the lower half.

The growth-promoting activity of a given concentration of any substance added to the basal medium may be expressed as the average colony diameter obtained with a given strain at 40 hours incubation. For example, Strain 1 gave no isolated colonies on the basal medium, and on the same medium with the addition of 25 cc of "double strength" meat infusion per 100 cc, a series of over 60 determinations extending over a period of several months gave an average colony size of 2.3 ± 0.5 mm. The large variation in this case is due to the crowding effect of such large colonies.

Isolation of Essential Factor—Since beef heart and meat infusions proved to be poor starting materials for the isolation of the essential growth factor, a search was made for a better source. Yeast infusion was found to give almost as large colonies as did meat infusion, and horse red blood cell extract supported growth but the colonies never exceeded 1.0 mm in size no matter how high the concentration of red cell extract. Assay curves for beef heart infusion, yeast infusion, and red cell extract are shown in Fig 1. The curve for red cell extract has the form expected for the assay of a single factor, but with the other two the colony size increases with increasing concentrations at least up to 25 cc per 100 cc of medium (about 1.0 gm of solids in each case), and possibly beyond. This indication of the presence of additional growth factors in beef heart and yeast was also borne out by the fact that highly active preparations from these sources gave smaller colonies than the original materials did, and the most active preparations gave approximately the same size colonies as did the red cell extract.

Highly active preparations were obtained from all three sources by chemical fractionation methods. The best yeast and red blood cell preparations were about equally active and the beef heart preparation somewhat less so. The chemical properties of all three were similar. The peptide nature of the active factor was shown by (1) complete destruction of the activity by acid hydrolysis, and by deamination with nitrous acid, and (2) by all the other evidence including the presence of free acid and basic groups, electro-metric titration curves, and amino nitrogen values. A "carboxyl nitrogen" determination by the ninhydrin method (6) of the yeast preparation gave a value of 31 per cent of the total nitrogen as carboxyl nitrogen. In general, peptides give no carboxyl nitrogen with the exception of glutathione, which Van Slyke found to give a value of 33.3 per cent. This indicated the active factor to be glutathione and it was then found that both natural and synthetic glutathione completely replaced the essential growth factor. As is evident in Fig 1, glutathione duplicates the effect of the red blood cell extract but does not give nearly as large colonies as beef heart or yeast infusions, thus confirming the presence of additional stimulatory factors in meat and yeast infusions. Analysis for total glutathione by the titration method of Woodward and Fry (7) showed the most active yeast and red

blood cell preparations to consist of about 80 per cent of glutathione, and this substance was actually isolated from a yeast preparation. The assay values are also in agreement with the analytical figures, since the yeast and red blood cell preparations were only slightly less active than pure glutathione. There is thus no doubt that the essential growth factor for these exacting strains present in all three sources is glutathione.

The methods of purification need not be given in detail, since glutathione can be readily and quickly isolated from yeast by the method of Hopkins (8) as modified by Schroeder *et al* (9). They are briefly summarized below.

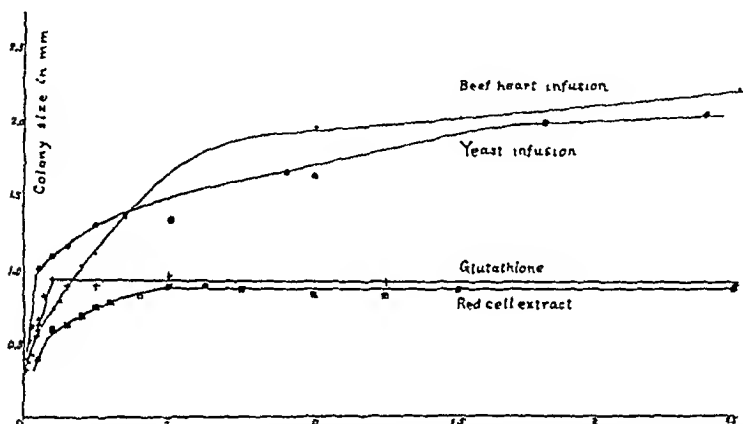


FIG 1 Assay curves for growth factors present in various preparations. The abscissa readings for the beef heart infusion, the yeast infusion, and the red blood cell extract are given in cc per 100 cc of medium, the glutathione, in mg per 100 cc of medium.

Beef Heart Infusion—(a) The infusion was treated with excess lead acetate and basic lead acetate solutions. The precipitate, after removal of the lead with H_2S and of acetic acid by concentration to dryness *in vacuo*, contained almost all of the original activity in the essential factor. (b) This material was then precipitated by excess mercuric acetate solution and the precipitate worked up in the same way as the lead precipitate. (c) Cold saturated $Ba(OH)_2$ was added to this solution until alkaline to phenolphthalein and the precipitate centrifuged and washed. This precipitate was completely inactive. The supernatant and washings were combined and treated with 4 volumes of alcohol, and the precipitate which formed was centrifuged and extracted with several small portions of water. The extracts, which contained the essential factor, were combined and all the Ba removed with a slight excess of H_2SO_4 . (d) This solution was then

treated with excess saturated alcoholic HgCl_2 and the precipitate worked up as for the Pb precipitate, except that the residue obtained was extracted with alcohol, which in the presence of the HCl left in the residue dissolved almost all of the essential factor. This material, which was probably as active as any of the other preparations from beef heart or meat, was used for the analysis given below. Almost 0.13 gm. was obtained from 2 liters of double strength meat infusion, representing about 2 kilos of meat.

Yeast—Yeast infusion was made by heating pressed bakers' yeast with an equal weight of 1 per cent acetic acid at $85-90^\circ$ for 15 minutes and filtering through kieselguhr.

The infusion was fractionally precipitated with three or four portions of mercuric acetate solution. The first precipitate usually contained most of the active material. This fraction was then precipitated with $\text{Ba}(\text{OH})_2$ as described for meat infusion. About 4.75 gm. were obtained from 5 kilos of yeast.

Further purification was obtained by precipitation with a large excess of AgNO_3 solution. After removal of Ag by treatment with H_2S and HCl the solution was concentrated to dryness *in vacuo* and the product used for assay and for glutathione analysis by titration.

Horse Red Blood Cells—After thorough washing with saline, the horse red blood cells were cytolysed and the proteins precipitated with acetone, and the acetone filtrate concentrated *in vacuo* as described by Warburg for the preparation of coenzyme II (10). This solution is the red blood cell extract mentioned above.

The solution was fractionally precipitated with mercuric acetate. Most of the activity was usually found to be in the third of the five fractions precipitated.

The most active fraction, after removal of Hg with H_2S , was concentrated to a small volume and precipitated with 4 volumes of acetone. The precipitate was rejected and the filtrate was concentrated to dryness *in vacuo* and the residue extracted with methyl alcohol. The filtered extract was treated with about 2 volumes of acetone and the precipitate rejected. The filtrates from several batches were combined at this stage and taken to dryness. The residue was extracted with ethyl alcohol and filtered and the filtrate evaporated to dryness. This material was then treated with $\text{Ba}(\text{OH})_2$, as described for yeast infusion. The resulting solution was used for the assay curves and analysis. A total of 290 mg. was obtained from about 3 liters of red blood cells.

The analytical results and assay values for the best preparations from all three sources and for pure glutathione are given in Table I. Although the theoretical amino nitrogen value for pure glutathione is 33 per cent, the actual value obtained is over 50 per cent (8).

It was found that some destruction of glutathione occurred during autoclaving. The minimum concentration that gave maximum colony size was 1.0 mg per cent if added before autoclaving and 0.6 mg per cent if added after, but, except where otherwise noted, all materials were added before autoclaving.

Glutathione is active in both the oxidized and reduced forms but its activity is completely destroyed by acid hydrolysis and by deamination. Cystine and cysteine not only will not replace it but are inhibitory to these strains, the amount required to inhibit growth being proportional to the amount of glutathione present. Approximately 3 molecules of cysteine will suppress the activity of 1 of glutathione. Since the casein hydrolysate contains some cystine (about 1.5 mg in 100 cc of medium), it appears probable that the glutathione requirement would be even smaller with a cystine-free medium.

TABLE I
Assay Values for Preparations Studied

Preparation	Total N	Amino N	Carboxyl N	Minimum concentration for optimum growth	Total GSH
	<i>per cent</i>	<i>per cent of total N</i>	<i>per cent of total N</i>	<i>mg per cent</i>	<i>per cent</i>
Glutathione	13.7	33 (Calculated) 50 (Found)	33.3	1.0	100
Beef heart infusion	12.5			(5.0?)	
Yeast infusion	13.5*	65*	31*	1.25	80
Red blood cell extract	12.8	75		1.10 (Estimated)	85

* These values were obtained on material before the last step in the purification.

Of a large number of substances tested for their ability to replace this peptide, only two have been found to have any activity whatsoever. Asparthione,² the aspartic acid analogue of glutathione, had some growth-promoting effect, although it did not give as good growth as glutathione. Isoglutathione, in which the glutamic acid is bound to cysteine through its α - rather than its γ -carboxyl group, appeared to have a very slight activity. Cystinyldiglycine was inhibitory and no other cysteine peptides have as yet been tested. In this connection it is of interest to note that Behrens (11) has recently reported that asparthione and isoglutathione have glyoxalase coenzyme activity, although asparthione is less active and isoglutathione very much less active than glutathione.

The principal function that has been suggested for glutathione is that of

* We wish to express our thanks to Dr. Vincent du Vigneaud for generously supplying us with the samples of synthetic glutathione, asparthione, isoglutathione, and cystinyldiglycine used in this study.

the coenzyme of glyoxalase and the above evidence suggests that it may be acting in that capacity in the growth of these gonococcus strains

Strains—A total of six glutathione-dependent strains have been encountered. Two of them were stock strains from this laboratory, one was a stock strain sent to us by Dr C Philip Miller, and the other three strains were originally not dependent on glutathione when freshly isolated but changed after some weeks of daily subculturing in the laboratory. Two of the stock strains and one which has changed were studied in detail and assay curves for two of these strains are given in Fig 2. In this case a sterile glutathione solution was added to the medium after autoclaving. Both strains gave a maximum colony size, at a glutathione concentration of 0.6 mg per 100 cc of medium, within the experimental error.

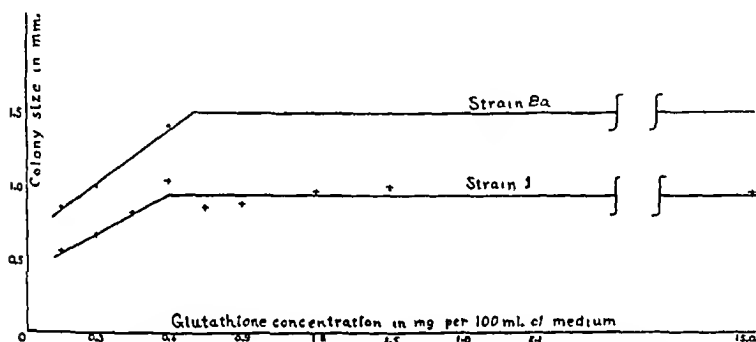


FIG 2 Assay curves for two glutathione dependent strains

DISCUSSION

No freshly isolated strains have been found to be dependent on glutathione, so it seems probable that this characteristic is an adaptation to continued subculturing on a medium rich in glutathione. The evidence indicates that most if not all gonococcus strains will change to a glutathione-dependent variant, although the time required may be a matter of chance and vary considerably. The strain which has been studied in the most detail in this regard, Strain 8, changed suddenly and completely but other strains have been encountered which showed a partial dependence on glutathione. They were in general capable of growth with the formation of very small colonies on the basal medium and were greatly stimulated by the addition of glutathione. Cystine (or cysteine) alone did not stimulate their growth but, when added to glutathione, the inhibition of growth was not as marked as with strains completely dependent on glutathione. Their behavior suggested that they were still capable of synthesizing glutathione but not at a sufficiently rapid rate for optimum growth. The glutathione-dependent variant is denoted as Strain 8a.

The inhibition of these strains by cystine appears similar to the inhibition of the growth of bacteria requiring *p*-aminobenzoic acid by sulfanilamide. Since these strains are unable to synthesize glutathione from cysteine, the presence of the latter may cause blocking of some essential metabolic process by competition with glutathione. However, as the gonococcus is inhibited by a great variety of substances, this explanation is only in the nature of a speculation.

Preliminary experiments indicate that the ability to grow without glutathione may be regained by a strain which has lost this ability. One strain, No. 8, previously discussed, which suddenly lost the ability to grow without glutathione when streaked out from a saline suspension by the standard technique, was subcultured on such a medium by the use of a very heavy inoculum. After several weeks of daily subculturing it was found that it gave the same size colonies without glutathione as when freshly isolated. These changes are as follows:

	Medium used for daily subcultures	Growth on basal medium (no glutathione)
Jan 29-Apr 6	Meat infusion	Jan 29-Mar 4 1.5 mm Mar 4-Apr 6 No growth
Apr 6-May 28	Basal medium	May 28 About 1.5 mm

SUMMARY

1 The factor present in meat infusion, yeast infusion, and red blood cell extract which is essential for the growth of certain stock strains of gonococcus has been shown to be glutathione.

2 A quantitative method of assay of growth factors has been developed for use on solid media, based on determination of the average colony size under standard conditions.

3 Freshly isolated strains have been found not to require glutathione but they show a tendency to develop a dependence on glutathione after some weeks of subculturing on a medium containing meat infusion.

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THE INFLUENCE OF GOITROGENIC SUBSTANCES ON THE CONVERSION IN VITRO OF INORGANIC IODIDE TO THYROXINE AND DIIODOTYROSINE BY THYROID TISSUE WITH RADIOACTIVE IODINE AS INDICATOR*

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It has long been known that the continued feeding of diets rich in certain plant materials causes hypertrophy and hyperplasia of the thyroid gland. The production of goiters in rabbits fed a diet high in cabbage leaves was first observed by Chesney and coworkers (1-3) and confirmed by Marine *et al* (4). In 1936 Hercus and Purves reported that the feeding of *Brassica* seeds led to thyroid hyperplasia in rats (5), this effect has been extensively studied by Kennedy, Griesbach, and Purves (6-8). In attempting to determine the causative factor in such seeds, Kennedy found that thyroid glands of rats that had received allylthiourea were markedly hypertrophied and hyperplastic (9). A similar change in the thyroid glands of rats fed phenylthiourea has been observed by Richter and Chisby (10). A detailed study of the goitrogenic activity of thiourea and its derivatives has been made recently by MacKenzie and MacKenzie (11) and by Astwood *et al* (12, 13).

Among the other substances reported to cause hypertrophy and hyperplasia of the gland are the cyanides, thiocyanate, and the sulfonamides. The effects of the first of these have been observed by Marine *et al* (14). The goitrogenic properties of thiocyanate have been repeatedly observed in patients who received this compound for treatment of hypertension (15). An extensive investigation of the goitrogenic action of these compounds has been made by MacKenzie and MacKenzie (11) and Astwood *et al* (12, 13).

The hypertrophy and hyperplasia of the thyroid gland produced by these substances are not due to a *direct* stimulation of thyroid tissue, since their goitrogenic effects are not observed in the hypophysectomized animal, enlargement of the gland in the intact animal is believed to occur in response to increased thyrotropic hormone activity. This indirect mechanism for the enlargement of the gland was first established for *Brassica* seeds by Griesbach *et al* (7, 8) and later shown to be true for the sulfonamides and thioureas by MacKenzie and MacKenzie (11) and Astwood *et al* (12). The latter (12) also found that the feeding of thiourea depressed the oxygen consumption of the intact animal but did not interfere with the calorigenic

* Aided by grants from the Commonwealth Fund and the Committee for Research in Endocrinology of the National Research Council

action of administered thyroxine or desiccated thyroid, they therefore concluded that thiourea and related compounds depress the production of the thyroid hormone. These observations have led to the introduction recently of thiourea and thiouracil in the treatment of hyperthyroidism (16).

The development of an *in vitro* reaction for measuring the conversion of radioactive inorganic iodide to thyroxine and diiodotyrosine by surviving thyroid tissue (17) provides a method for determining the action of these goitrogenic compounds. In respect to the sulfonamides, it was shown previously that at a concentration of 10^{-3} M they interfere markedly with the conversion of inorganic iodide to thyroxine and diiodotyrosine but not appreciably with the iodine-concentrating capacity of the surviving thyroid slices (18, 19). The present report deals with the action of methyl cyanide, thiourea, allylthiourea, thiouracil, thiocyanate, *p*-aminobenzoic acid, and *p*-aminophenylacetic acid.

Results

Formation of Radiothyroxine and Radiodiiodotyrosine

Thiourea, Thiouracil, and Allylthiourea—The effects of these three compounds are shown in Table I. Thiourea and thiouracil inhibited the conversion of the radioactive iodine of the Ringer's solution to thyroxine and diiodotyrosine at a concentration as low as 10^{-3} M. Allylthiourea depressed this conversion markedly even at a concentration of 10^{-4} M (0.001 per cent solution).

Methyl Cyanide—The effects of two concentrations of this organic cyanide are shown in Table II. No interference in the formation of radiothyroxine or radiodiiodotyrosine was observed when the Ringer's solution contained 0.04 per cent (10^{-2} M) of this compound. It did not inhibit the formation of thyroxine and diiodotyrosine even at a concentration of 10^{-1} M.¹

Potassium Thiocyanate—A marked decrease in the amount of the radioactive iodine of the Ringer's solution converted to thyroxine and diiodotyrosine was found when thiocyanate was present in the medium at concentrations of 10^{-2} and 10^{-3} M (Table II).

***p*-Aminobenzoic Acid and *p*-Aminophenylacetic Acid**—The goitrogenic properties of these two compounds were demonstrated by Astwood (13). In keeping with this finding, Table II shows that their presence in the Ringer's medium decreased the formation of radiothyroxine and radiodiiodotyrosine.

Penetration of Inorganic Iodide I^{131} into Thyroid Slices

In the experiments of Tables I and II, the contents of the entire reaction flask (i.e. slices as well as the 3 cc. of medium) were analyzed for thyroxine

¹ Values for radiothyroxine and radiodiiodotyrosine obtained at this concentration are not recorded in Table II.

and diiodotyrosine In the experiments of Tables III to V, the thyroid slices were separated from their medium and only the slices analyzed for either total I^{131} or organic I^{131} and inorganic I^{131} In this way the effect of each goitrogenic compound on the iodine-concentrating capacity of thyroid tissue was determined

TABLE I

Effect of Thiourea, Thiouracil, and Allylthiourea on Formation of Radiothyroxine and Radiodiiodotyrosine by Surviving Sheep Thyroid Slices

300 mg of slices were incubated for 2 hours at 38° in a bicarbonate-Ringer's medium containing inorganic iodide, I^{131} No I^{127} was added *

Experiment No	Compound added	Concentration	Radioactive iodine of Ringer's solution recovered as	
			Thyroxine	Diiodotyrosine
		<i>M</i>	<i>per cent</i>	<i>per cent</i>
1	None	Control	9.6	35.3
	"	"	10.6	37.6
	Thiourea	10^{-2}	0.2	2.1
	"	10^{-2}	0.2	1.9
	"	10^{-3}	0.9	1.6
	"	10^{-3}	0.8	2.4
	Allylthiourea	10^{-2}	0.2	2.3
	"	10^{-2}	0.3	2.9
	"	10^{-3}	0.3	2.4
	"	10^{-3}	0.3	2.8
	"	10^{-4}	1.1	4.6
2	"	10^{-4}	0.9	3.4
	None	Control	8.8	73.3
	"	"	8.8	64.6
	Thiourea	10^{-2}	0.6	6.0
	"	10^{-3}	0.6	7.3
	Allylthiourea	10^{-3}	0.3	9.9
	"	10^{-3}	0.4	6.5
	Thiouracil	10^{-2}	0.3	5.2
	"	10^{-2}	0.3	7.2

* The only I^{127} contained in the reaction flask was that due to impurities in the reagent quality chemicals used in the preparation of the media

In the experiments of Table III, 300 mg of thyroid slices were transferred to a Ringer's medium containing both the compound to be tested and the radioactive iodide and incubated at 38° for 2 hours Up to this point the procedure was the same as in the experiments of Tables I and II The slices were then removed and, in order to free the slices of I^{131} adhering to their surfaces, washed twice, each time for 20 seconds in 3 cc of a non-radioactive Ringer's solution The slices were then analyzed for total I^{131}

Controls—In control experiments in which none of the goitrogenic sub-

stances were added to the Ringer's medium, 90 per cent or more of the I^{131} that had been added to the Ringer's medium was recovered in the thyroid slices after their incubation for 2 hours. These values should be contrasted

TABLE II

Effect of Methyl Cyanide, Potassium Thiocyanate, p-Aminobenzoic Acid, and p-Aminophenylacetic Acid on Formation of Radiothyroxine and Radiodiiodotyrosine by Surviving Sheep Thyroid Slices

300 mg. of slices were incubated for 2 hours at 38° in a bicarbonate-Ringer's medium containing inorganic iodide, I^{131} . No I^{-} was added.*

Experiment No.	Compound added	Concentration	Radioactive iodine of Ringer's solution recovered as	
			Thyroxine	Diiodotyrosine
1	None	Control	9.6	35.3
	"	"	10.6	37.6
	Methyl cyanide	10^{-3}	11.1	31.7
	" "	10^{-3}	11.1	34.1
	" "	10^{-2}	10.4	30.0
	" "	10^{-2}	11.3	37.6
2	None	Control	8.8	73.3
	"	"	8.8	64.6
	Methyl cyanide	10^{-3}	8.2	68.4
	" "	10^{-3}	8.1	74.1
	Potassium thiocyanate	10^{-3}	3.5	30.6
	" "	10^{-3}	0.8	10.6
	p-Aminobenzoic acid	10^{-3}	1.0	5.9
	" "	10^{-3}	0.8	6.2
	p-Aminophenylacetic acid	10^{-3}	1.3	5.5
3	" "	10^{-3}	1.3	7.5
	None	Control	12.1	51.9
	"	"	12.4	56.9
	Potassium thiocyanate	10^{-3}	0.8	16.0
	" "	10^{-3}	0.9	14.3
4	None	Control	5.8	49.2
	"	"	5.1	37.4
	"	"	5.6	46.2
	p-Aminobenzoic acid	10^{-3}	0.9	4.3
	" "	10^{-3}	1.1	
	" "	10^{-3}	1.0	3.8

* See the foot-note to Table I

with those obtained when 300 mg. of liver slices were incubated under identical conditions, as much as 10 per cent of the radioactive iodine of the Ringer's solution was recovered in these slices (19)

Thiourea—A concentration of thiourea that almost completely depressed

the formation of radiothyroxine and radiiodotyrosine had relatively little effect on the uptake of I^{131} by the thyroid tissue. In Experiment 3 (Table III) the recovery of I^{131} in the presence of 10^{-3} M thiourea was about 10 per cent less than in the controls, in Experiment 1 the 300 mg of thyroid slices had accumulated approximately 80 per cent of the radioactive iodine of the Ringer's solution in the presence of this concentration of thiourea.

TABLE III

Effect of Gastrogenic Compounds on Accumulation of Inorganic Iodide I^{131} by Surviving Sheep Thyroid Slices

300 mg of slices were incubated for 2 hours at 38° in a bicarbonate-Ringer's medium containing inorganic iodide, I^{131} . No I^{127} was added.* The slices were then separated from the medium and analyzed.

Experiment No	Compound added	Concentration	Radioactive iodine of Ringer's solution recovered in thyroid slices
			per cent
1	None	Control	93
	"	"	90
	Thiourea	10^{-3}	70
	"	10^{-3}	79
	Potassium thiocyanate	10^{-3}	46
	"	10^{-3}	55
	p-Aminobenzoic acid	10^{-3}	83
2	"	10^{-3}	90
	None	Control	88
	"	"	85
	Thiouracil	10^{-3}	79
	"	10^{-3}	81
3	None	Control	97
	"	"	98
	Thiourea	10^{-3}	90
	"	10^{-3}	89
	p-Aminobenzoic acid	10^{-3}	89
	"	10^{-3}	84
	Potassium thiocyanate	10^{-3}	50
	"	"	61
	"	10^{-2}	5
	"	10^{-2}	8

* See the foot-note to Table I

Thiouracil—In the experiments of Table III, "tracer" amounts of I^{131} were added to the Ringer's solution, the only I^{127} present in the reaction flask was that due to the impurities in the reagents used in the preparation.

* The saline solution containing the radioiodide (I^{131}) as prepared from the tellurium target contained an amount of iodine ($s_2I^{127} + s_2I^{131}$) that was not detectable chemically.

of the Ringer's medium. In the experiments of Table IV, 10 γ of I^{127} as KI were added to each 3 cc of Ringer's solution. Since the ratio of I^{131} to I^{127} (specific activity) in the medium was known, the recovery of I^{131} in the thyroid slices also is a measure of the amount of I^{127} of the Ringer's solution that entered the thyroid slices. The micrograms of I^{127} that entered the thyroid slices during a 1 hour period of incubation are recorded in the last three columns of Table IV.

A concentration of 10^{-3} M thiouracil had little effect on the uptake of radioiodide by thyroid slices when tracer amounts of I^{131} were added to the incubation medium (Table III).

Table IV shows in a most striking manner that, while depressing the formation of thyroxine and diiodotyrosine, thiouracil at a concentration of

TABLE IV
*Effect of Thiouracil on Accumulation of I^{127} of Ringer's Solution
by Surviving Thyroid Slices*

300 mg of slices were incubated for 1 hour at 38° in a bicarbonate-Ringer's medium containing inorganic iodide, I^{131} , and 10 γ of inorganic iodide, I^{127} . The slices were then separated from their medium and analyzed.

Compound added	I^{131} of Ringer's solution recovered in slices as			I^{127} of Ringer's solution incorporated into slices as		
	Organic	Inorganic	Total	Organic*	Inorganic	Total
	per cent	per cent	per cent	γ	γ	γ
None	26.4	56.2	82.6	2.64	5.62	8.26
"	22.9	50.0	72.9	2.29	5.00	7.29
"	13.6	54.0	67.6	1.36	5.40	6.76
10^{-3} M thiouracil	2.4	75.5	77.9	0.24	7.55	7.79
10^{-3} " "	2.3	65.2	67.5	0.23	6.52	6.75
10^{-3} " "	2.0	59.8	61.8	0.20	5.98	6.18

* Refers to thyroxine-like and diiodotyrosine-like iodine (see the text)

10^{-3} M has no effect on the absolute amounts of I^{127} removed from the Ringer's medium by the thyroid slices. Thus 7 to 8 γ of I^{127} were recovered in the thyroid slices when 10 γ of I^{127} were added to the medium, of this, 1.4 to 2.6 γ were converted to thyroxine and diiodotyrosine. When thiouracil as well as 10 γ of I^{127} were added to the Ringer's medium, 6 to 8 γ of I^{127} entered the 300 mg of thyroid slices, practically none of it, however, was converted to thyroxine and diiodotyrosine in 1 hour.

p-Aminobenzoic Acid—The effect of this compound on the penetration of I^{131} was tested at a single concentration, namely, 10^{-3} M. In Experiment 1 (Table III) little difference in I^{131} uptake was observed whether or not the thyroid slices were treated with this compound. In Experiment 3, the uptake was depressed only about 10 per cent by *p*-aminobenzoic acid.

Potassium Thiocyanate—An appreciable reduction in the uptake of I^{131}

was observed in the presence of 10^{-3} M thiocyanate. At this concentration, however, considerable amounts of the I^{131} added to the Ringer's medium were recovered in the thyroid slices after their incubation for 2 hours. Increasing the thiocyanate to 10^{-2} M completely abolished the iodine-concentrating capacity of this tissue. The amounts of I^{131} that entered the slices incubated in 10^{-2} M thiocyanate were about the same as those found when liver was used (19).

Although the conditions in which penetration was studied in the experiments of Table III were the same as those of the experiments of Tables I

TABLE V

Effect of Goitrogenic Compounds on Accumulation of Inorganic Iodide I^{131} by Surviving Thyroid Slices

300 mg of slices were first treated for 30 minutes at 38° in a non-radioactive bicarbonate-Ringer's medium containing one of the goitrogenic compounds, the slices were then removed and transferred to bicarbonate-Ringer's medium containing the same goitrogenic compound and inorganic iodide, I^{131} , and incubated for 2 more hours at 38° . No I^{127} was added during the first or second incubation.*

Experiment No	Compound added	Concentration	Radioactive iodine of Ringer's solution recovered in slices
			per cent
1	None	Control	88
	"	"	92
	Thiouracil	10^{-3}	77
	"	10^{-3}	82
2	None	Control	93
	"	"	94
	Thiourea	10^{-3}	88
	"	10^{-3}	91
	Potassium thiocyanate	10^{-3}	39
	" "	10^{-3}	36
	p-Aminobenzoic acid	10^{-3}	94
	" "	10^{-3}	84

* See the foot-note to Table I

and II, in which the formation of thyroxine and diiodotyrosine was measured, it might be thought unjustified to conclude that thiourea, thiouracil, and p-aminobenzoic acid do not appreciably alter the iodine-concentrating capacity of thyroid slices, since the I^{131} probably enters the slices faster than these three goitrogenic compounds. To investigate this possibility, experiments were carried out in which thyroid slices were pre-treated with these compounds before they were incubated with radioactive iodine.

In the experiments of Table V, 300 mg of thyroid slices were incubated

at 38° for 30 minutes in a non-radioactive Ringer's solution containing either thiouracil or thiourea or potassium thiocyanate or *p*-aminobenzoic acid. After this preliminary incubation, the slices were carefully removed from the reaction flask, deposited for about 10 seconds on filter paper previously moistened with Ringer's solution, and then transferred to a flask in which the medium contained the radioactive iodide and the same concentration of the same goitrogenic compound to which the slices had already been exposed. The incubation of the slices was then continued for 2 hours. The results obtained in this type of penetration study did not differ from those recorded in Table III. Thiouracil, thiourea, and *p*-aminobenzoic acid at a concentration of 10^{-3} M had little effect on the uptake of I^{131} , whereas this concentration of thiocyanate reduced the iodine-concentrating capacity of the thyroid slices to about half of that found in the controls.

DISCUSSION

It is shown here that thiourea, thiouracil, and allylthiourea inhibit the conversion of inorganic iodide of the Ringer's solution to thyroxine and diiodotyrosine by surviving thyroid tissue. The extreme sensitivity of the reaction here involved to compounds of this type is well brought out in the case of allylthiourea, this substance markedly depressed the formation of radiothyroxine and diiodotyrosine even at a concentration as low as 10^{-4} M. *p*-Aminobenzoic acid and *p*-aminophenylacetic acid had a pronounced effect on the rate of conversion of inorganic iodide to thyroxine and diiodotyrosine. These two compounds have been shown to be interchangeable as essential bacterial metabolites (20). Their use in the present investigation was suggested not only by the fact that they possess goitrogenic activity (13) but also because of their structural similarity to the sulfonamides, which in an earlier study from this laboratory were shown to depress the conversion of inorganic iodide to thyroxine and diiodotyrosine (18, 19). It is of interest to note here that *p*-aminobenzoic acid influences oxidative processes catalyzed by tyrosinase. Wisansky *et al* reported that the aerobic oxidation of tyrosine is retarded by *p*-aminobenzoic acid (21). It was shown earlier in this laboratory that the formation of both diiodotyrosine and thyroxine by the thyroid gland is linked with aerobic oxidations in which the cytochrome-cytochrome oxidase system is involved (22).

Astwood found that in the intact rat the administration of sufficient inorganic iodide prevented the goitrogenic effects of thiocyanate but not of thiourea or the sulfonamides (12, 13). This *in vivo* difference may find an explanation in the *in vitro* observations made in this laboratory. When comparisons were made at a single concentration, namely 10^{-3} M, it was found that the action of thiocyanate differed from that of thiourea, thi-

ouracil, and *p*-aminobenzoic acid. The three latter compounds strongly inhibited the conversion of the radioactive iodine of the Ringer's solution to thyroxine and diiodotyrosine, but had little effect on the amount of I^{131} accumulated by the thyroid slice, 10^{-3} *M* sulfanilamide also depressed the formation of radiothyroxine and radiodiiodotyrosine without appreciably influencing the iodine-concentrating capacity of thyroid slices (19). Thiocyanate, on the other hand, decreased the amount of I^{131} that entered the slice as well as the amount recovered as thyroxine and diiodotyrosine.

It is interesting to mark the close interrelation that exists among three types of compounds studied here: the cyanides, thiocyanate, and the thioureas. In the animal body, the cyanides (both inorganic and the nitriles) are converted to thiocyanate (23, 24), whereas in the test-tube thiocyanate and thiourea are interconvertible (25, 26). The extent to which such conversions are involved in the goitrogenic effects produced by the feeding of these three types of compounds is, of course, not known at present.

It was shown previously in this laboratory that NaCN strongly inhibits the *in vitro* conversion of inorganic iodide to thyroxine and diiodotyrosine by surviving thyroid slices (22). Hence the failure of methyl cyanide (Table II) to retard this conversion even at a concentration as high as 10^{-1} *M* suggests that thyroid hyperplasia, when produced in the intact animal by the administration of methyl cyanide (14), is not the result of the action of the CH_3CN molecule *per se* upon thyroid tissue but of its detoxification product, thiocyanate, or its hydrolysis product, cyanide.

EXPERIMENTAL

Preparation of Media—The bicarbonate-Ringer's medium used here was prepared according to Krebs and Henseleit (27). Radioactive inorganic iodide (I^{131}) was added as a solution of isotonic NaCl, the volume added to the bicarbonate buffer was always small in comparison with the total volume of the medium. The preparation of the radioiodine has been described elsewhere (28). No I^{127} was added to the media used in the experiments of Tables I, II, III, and V, hence the only I^{127} present in the media of these experiments was that due to impurities in the reagent grade chemicals used in preparation of the media.

3 cc portions of this radioactive buffer were transferred to each reaction flask (25 cc Erlenmeyer flask). The various compounds shown in Tables I to V were then added in 0.1 cc of isotonic NaCl. The compounds were prepared of such strength that the addition of 0.1 cc to the 3 cc medium yielded the concentrations of the compounds recorded in these tables. The pH of the concentrated solutions of the compounds in isotonic NaCl was adjusted to 7.4 before their addition to the bicarbonate-Ringer's me-

dium In the control experiments, 0.1 cc of isotonic NaCl containing none of these compounds was added to 3 cc of the radioactive buffer The thiouracil was supplied by the Lederle Laboratories, Inc., to whom our thanks are due All other compounds used were of the c.p. grade

Preparation of Tissue Slices and Their Incubation—The thyroid glands were obtained at the local abattoir from sheep, weighing approximately 30 kilos, that were sacrificed by exsanguination The glands were immediately wrapped in cellophane, packed in ice, and brought to the laboratory About 1 hour elapsed between the time the sheep were killed and the slicing of the glands was begun

The preparation of the thyroid slices has been described elsewhere (17) 300 mg of slices were transferred to each reaction flask containing the prepared medium The bicarbonate-Ringer's solution was saturated with a gas mixture consisting of 95 per cent O_2 and 5 per cent CO_2 before the addition of the radioiodide, and the atmosphere in the flask above the liquid was flushed with the same gas mixture immediately after the addition of the thyroid slices The flasks were then tightly stoppered and incubated at 38° They were gently agitated during the entire period of incubation

Separation of Thyroxine, Diiodotyrosine, and Inorganic Iodide in Experiments of Tables I and II—In the experiments of Tables I and II the entire contents of each flask, i.e. slices as well as the medium, were analyzed for radiothyroxine, radiodiiodotyrosine, and inorganic I^{131} At the end of the incubation period, 3 cc of 4 N NaOH were added to each flask and the contents hydrolyzed for 8 hours on a steam bath The further separation was carried out after the manner described by Perlman *et al.* (29)

Penetration Studies of Tables III and V—The slices were separated from their medium in the following manner The liquid in each flask was decanted The slices were then immersed for 20 seconds in 3 cc of a non-radioactive bicarbonate-Ringer's solution This was repeated for another 20 seconds in a fresh 3 cc portion of non-radioactive bicarbonate-Ringer's solution The slices were then placed on filter paper moistened with the bicarbonate-Ringer solution in order to remove excess liquid adhering to the surfaces of the slices The thyroid slices were then transferred to a flask containing 6 cc of 2 N NaOH and hydrolyzed until a homogeneous solution was obtained

Penetration Study of Table IV—The thyroid slices were separated from the medium in which they were incubated and washed as described above They were then transferred to a flask containing 6 cc of 2 N NaOH and hydrolyzed for 8 hours on a steam bath The procedure employed here for the determination of inorganic I^{131} and organic I^{131} is a modification of that used in the experiments of Tables I and II

Radioactive inorganic iodide was separated from radiothyroxine and

radioduodotyrosine in the following manner 0.1 cc of 0.2 M KI was added as carrier to the above alkaline hydrolysate and the solution made acid to methyl orange with H_2SO_4 . The iodide was oxidized with iodate and the newly formed iodine (I_2) extracted with carbon tetrachloride. Radiothyroxine and radioduodotyrosine were contained in the aqueous phase. This procedure differed from that used in Tables I and II in that the initial removal of thyroxine with butyl alcohol was not carried out, the oxidation of the iodide by means of iodate being carried out in the presence of thyroxine. This procedure yielded two fractions, one of which contained the inorganic iodide I^{131} and the other the I^{131} that could not be oxidized to I_2 with iodate, the latter contained the organically bound I^{131} of the hydrolysate.

Determination of Radioactivity—Aliquots of the various fractions were transferred to Coors milk-ashing dishes and evaporated to dryness. The presence of a small piece of lens paper insured an even distribution of the solid material on the bottom of the dish. A few drops of 2 N NaOH were added to prevent loss of iodine during the evaporation. The radioactivity was measured by means of a scale-of-eight Geiger-Muller counter. The tube contains a thin mica window (30).

SUMMARY

In order to study the mechanism of action of various known goitrogenic compounds, their effects on the *in vitro* conversion of radioactive inorganic iodide (I^{131}) to thyroxine and duodotyrosine by surviving thyroid slices as well as their effects on the uptake of total I^{131} by the slices were measured.

1 Thiourea, thiouracil, and allylthiourea strongly depressed the conversion of the medium's inorganic iodide to thyroxine and duodotyrosine. Allylthiourea was an effective inhibitor at a concentration as low as 10^{-4} M.

2 Methyl cyanide had no effect on the conversion of the medium's inorganic iodide to thyroxine and duodotyrosine even at a concentration as high as 10^{-1} M.

3 Thiocyanate inhibited the formation of radiothyroxine and radioduodotyrosine at a concentration as low as 10^{-3} M.

4 *p*-Aminobenzoic acid and *p*-aminophenylacetic acid depressed the conversion of the inorganic iodide of the Ringer's solution to thyroxine and duodotyrosine at a concentration of 10^{-3} M.

5 At a concentration that strongly inhibited the formation of radiothyroxine and radioduodotyrosine at the expense of the inorganic iodide of the medium, namely 10^{-3} M, thiourea, thiouracil, and *p*-aminobenzoic acid had little effect on the iodine-concentrating capacity of surviving thyroid slices. At this concentration thiocyanate depressed the uptake of radioactive iodide by thyroid slices as well as its subsequent conversion to thyroxine and duodotyrosine.

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THE DETERMINATION OF URIC ACID IN URINE WITH CRUDE URICASE*

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Blauch and Koch (1) introduced the uricase method for the determination of blood uric acid. This was based on the destruction of uric acid by crude uricase, so that the difference in phosphotungstate color before and after uricase action was assumed to be a measure of the true uric acid.

The uricase method has not been applied to human urine. This is of interest as a new means of checking the specificity of current methods for uric acid in urine. Folin (2) and Christman and Ravitch (3), using indirect methods for the determination of uric acid in urine, *i.e.* those involving the preliminary separation of uric acid before its colorimetric estimation, found that about 90 to 95 per cent of the value obtained by direct methods was true uric acid.

In this report the uricase method has been applied to the determination of uric acid in urine and the results so obtained have been compared with those by Folin's direct method (2, 4).

Methods

The true uric acid of urine determined by the uricase method was the difference between the value obtained by Folin's direct method (2, 4) and the color value for non-uric acid which remained after incubation of the urine with crude uricase.

In order to obtain the color value for the non-uric acid, the urine was diluted to a uric acid concentration of about 0.04 mg per cc. A preliminary direct uric acid determination was done on urine diluted 1:100 or 1:200 and on the basis of this a new dilution to 0.04 mg per cc was made.¹ 2 cc of the diluted urine were added to a 50 cc Erlenmeyer flask, followed by 50 mg of crude uricase powder (1). The flask was tightly stoppered, gently whirled, and incubated in an oven or water bath at 40–48° for 2 hours. Then 2 cc of water, 0.5 cc of 10 per cent sodium tungstate, and 0.5 cc

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¹ With ordinary urine containing 0.3 to 0.6 mg of uric acid per cc a 1:10 dilution would be satisfactory.

of $2/3$ N sulfuric acid were added to the flask, followed by shaking and filtering

To obtain the uric acid of the urine by Folin's direct method the urine was diluted to about 0.004 mg of uric acid per cc on the basis of the preliminary determination. If protein was present, it was removed from the original urine by acetic acid and heat.

The color was developed by the Folin method (2, 4). A Klett-Sumnerson photoelectric colorimeter with Klett tubes graduated at 5 and 10 cc was used. 1 cc samples were taken for all determinations. 1 cc of diluted uric acid standards were also taken. With the direct method these contained 0.003, 0.004, or 0.006 mg of uric acid per cc and were prepared by dilution of the stock standard (5) with water. 1 cc of water was added to another tube for a blank determination on the Folin reagents. 2 cc of the urea-cyanide solution were added to each tube, mixed, and followed by 0.8 cc of the concentrated uric acid reagent. After standing for 20 to 40 minutes, the solutions were diluted to 10 cc, mixed, and read.

The non-uric acid color was similarly developed, except that a 0.0004 mg or other suitable uric acid standard was used and the final volume was made to 5 cc. The same quantities of Folin reagents were taken as when the final volume was made to 10 cc.²

The unknowns were always read against the standard that matched them most closely. Since the uric acid color was not perfectly stable, the blank solution was read instead of adjusting the zero position of the galvanometer with it. When there were a number of unknowns, the blank and standard were read before, after, and sometimes in the middle of the unknowns.

The urine in the present study was partly from normal subjects and partly from patients with preeclampsia.

Results

The uric acid values of fifteen specimens of human urine, as determined by both Folin's direct and the uricase methods as described, are given in Table I. The true uric acid determined by the uricase method varied from 12.9 to 93.3 mg per cent. This was 94 to 98 per cent and averaged 96.5 per cent of that by Folin's direct value. The non-uric acid color after uricase action varied from 0.2 to 2.8 mg per cent and averaged 1.5 mg per cent, expressed as uric acid.

That uric acid added to urine was destroyed when incubated with uricase is shown in Table II. A solution of sodium or lithium urate was added

To reduce the size of the blank the use of 1 cc of urea-cyanide solution and 0.4 cc of concentrated uric acid reagent with the 5 cc volume is suggested. The uricase powder may give a small blank. If so, it should be subtracted from the readings of the unknown.

to urine of known true uric acid content The calculated total true uric acid of the mixture was compared with that determined from its color value for non-uric acid The uric acid destruction in three experiments was 100, 97, and 101 per cent

TABLE I
Uric Acid in Urine

Urine specimen No	Folin's direct method	Non uric acid color* after uricase	True uric acid	True uric acid Folin's direct method
	mg per cent	mg per cent	mg per cent	per cent
1	35.4	2.3	33.1	94
2	42.7	2.5	40.2	94
3	52.4	2.8	49.6	95
4	40.7	2.1	38.6	95
5	18.4	0.8	17.6	96
6	34.9	1.4	33.5	96
7	95.8	2.5	93.3	97
8	34.1	1.0	33.1	97
9	31.2	1.0	30.2	97
10	26.1	0.7	25.4	97
11	66.3	2.1	64.2	97
12	75.6	1.7	73.9	98
13	36.5	0.7	35.8	98
14	17.9	0.3	17.6	98
15	13.1	0.2	12.9	98
Average		1.5		96.5

* Expressed as uric acid

TABLE II
Destruction of Uric Acid Added to Urine When Incubated with Crude Uricase

True uric acid* of urine	Uric acid* added	True uric acid of urine + uric acid		Uric acid destruction
		Found	Calculated	
mg per cent	mg per cent	mg per cent	mg per cent	per cent
1.53	1.46	2.99	2.99	100
2.17	1.80	3.83	3.97	97
1.51	1.78	3.33	3.29	101

* The concentration present in the mixture of urine + uric acid

If there was any decrease of the color value for the non-uric acid of urine under the conditions of the uricase incubation, it would make the true uric acid value too high This was investigated on nine specimens of urine by determining the uric acid value by Folin's direct method before and after incubation without uricase After incubation the uric acid

values were from 6.6 per cent lower to 4.0 per cent higher and averaged 1.2 per cent lower than the initial values and therefore were not significantly changed.

Since the pH optimum for uricase action is 9.2, some of the acid urines were neutralized before they were diluted for incubation. However, with two urine specimens the same color value for non-uric acid was obtained on acid and neutralized samples.

DISCUSSION

The present findings are consistent with those of others (2, 3) using indirect precipitation methods for uric acid, that over 90 per cent of the uric acid in urine as determined by direct colorimetric methods is true uric acid. Moreover they indicate that about 95 per cent of the color value determined by Folin's direct method is true uric acid and that therefore it is a measure of true uric acid for all practical purposes.

The color determination for non-uric acid is subject to error owing to the very low concentrations measured and this in turn affects the true uric acid value, since the latter is determined by difference. However, a large error in this determination has only a small effect on the true uric acid value.

Inasmuch as crude uricase is used, the specificity of the present uricase method rests on the assumption that no color material other than uric acid is destroyed by it.

SUMMARY

A method is described for the determination of uric acid in human urine with crude uricase.

Uric acid determined on fifteen specimens of urine by the uricase method gave values from 94 to 98 per cent and averaged 96.5 per cent of those by the Folin direct method.

For all practical purposes Folin's direct method is an accurate means for the determination of true uric acid in urine.

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ON THE PURITY OF SYNTHETIC *dl*-LEUCINE

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Investigations in this and other laboratories have demonstrated that leucine and isoleucine are among the amino acids essential for *Lactobacillus arabinosus* (1-3). In an attempt further to purify our basal medium *dl*-leucine was used as a source of leucine rather than *l*(+)-leucine. To our surprise an isoleucine requirement could not be shown after this change was made. The *dl*-leucine satisfied both the leucine and isoleucine needs of the organism. This unexpected finding suggested that *l*(+)-leucine isolated from natural sources was free of isoleucine, but that the synthetic material contained either isoleucine or some material with isoleucine activity. This paper reports investigations upon the isoleucine activity of commercial *dl*-leucine samples and the identification of *dl*-isoleucine as a contaminant.

EXPERIMENTAL

The basal medium and assay techniques have been described (1). The standards used were *l*(+)-leucine (Merck) for the leucine assays and *dl*-isoleucine (Merck) for the isoleucine assays. Each sample of *dl*-leucine has been tested for isoleucine activity in at least two independent assays at four different levels.

The results obtained with seven different samples of *dl*-leucine are shown in Table I. Four of the seven samples showed unexpectedly high isoleucine activity and only two were apparently free of such activity. Samples 3 and 6 were assayed at levels up to 300 γ per tube. Sample 4 showed clear cut evidence of activity at this level but was finally tested at levels from 0.5 to 1.5 mg per tube. Samples of *d*(-)-leucine, tertiary leucine, and norleucine proved inactive, thus eliminating these isomers as a source of isoleucine activity.

Assays of these samples for *l*(+)-leucine are also shown. The activity of *d*(-)-leucine was surprising and appears to represent true activity, since rotation data indicate that not more than 1 or 2 per cent of *l*(+)-leucine could have been present in this sample. This material was of necessity tested at much higher levels than the *dl*-leucine samples. Recovery of *l*(+)-leucine added to tubes containing *d*(-)-leucine was not satisfactory if the leucine activity of the *d*(-)-leucine was taken as 9.2

per cent Investigations on the possible stimulatory action of *d*(-)-leucine when small amounts of *l*(+)-leucine are present are being continued

The leucine assays upon various leucine preparations are obviously a less accurate indication of the extent of contamination. However, Samples 1 and 5 have been assayed repeatedly and appear to be definitely less active than expected

Sample 1 has been studied by chemical and fractionation methods. Chromic acid oxidation by the procedure of Block and Bolling (4) demon-

TABLE I
Isoleucine and Leucine Activity of Samples of Commercial dl-Leucine and Leucine Isomers

Sample*	Activity Compared to <i>dl</i> isoleucine	<i>l</i> (+) Leucine content
	per cent	per cent
<i>dl</i> -Leucine, Sample 1	18.4	41.5
" " 2	16.8	45.0
" " 3	0.0	57.7
" " 4	0.51	
" " 5	6.78	32.0
" " 6	0.0	51.6
" " 7	12.2	
<i>d</i> -Leucine†	0.0	9.2
Tertiary leucine‡	0.0	
Norleucine	0.0	

* The seven samples of *dl*-leucine include products from four different commercial sources

† The authors are indebted to Dr. Sarah Ratner for a generous supply of *d*(-) leucine and for one of the *dl*-leucine samples. Concerning the sample of *d*(-) leucine, we quote, with permission, from a letter from Dr. Ratner, "The optical rotation of this sample indicates it to be the pure *d* variety, but since the rotation of leucine is rather low, the experimental conditions of estimation are such that the presence of 1 or 2 per cent of the *l* form cannot be excluded."

‡ The tertiary leucine was kindly supplied by Dr. David Bonner

strated the formation of considerably more methyl ethyl ketone than did a sample of *l*(+)-leucine

The copper salts of Sample 1 were prepared according to the procedure of Brazier (5). Cold water extraction appeared to remove only small amounts of material, and they were then extracted three times with hot water. After the removal of copper from the combined water extracts, isoleucine assay indicated that approximately 90 per cent of the nitrogen could be accounted for in this fraction as *dl*-isoleucine. Treatment of this fraction with *p*-toluenesulfonyl chloride yielded the tosyl derivative which,

upon recrystallization from benzene, melted at 139° (uncorrected) McChesney and Swann (6) record the melting point of tosyl-*dl*-isoleucine as 139-140° (uncorrected), and an authentic sample of tosyl-*dl*-isoleucine which was prepared melted at 139°. A mixed melting point of 139° identified the isolated material as *dl*-isoleucine¹

DISCUSSION

Isoleucine appears to be a common contaminant of synthetic *dl*-leucine. It should be stressed, however, that the data presented in this paper cannot be considered as representing the extent of impurity upon a *weight* basis. Data upon the specificity of the leucine and isoleucine requirements, to be published as a separate paper, have shown that *l*(+)-alloisoleucine has isoleucine activity but to a lesser extent than *l*(+)-isoleucine. Synthetic methods, such as described by Marvel (7), might be expected to give the four isomers in equal proportion, although we have no data on the origin of the materials or the methods used in *dl*-leucine manufacture. The isolation of pure *dl*-isoleucine as the tosyl derivative does not preclude the presence of alloisoleucines.

Investigators in biological fields are apt to consider synthetic chemicals as pure, at least in so far as other biologically active materials are concerned. These studies indicate the need for caution, especially when slight contamination may be of importance. It is of interest that Wood, Geiger, and Werkman (8) reported *dl*-leucine and *dl*-isoleucine to be interchangeable as nutrients for three species of heterofermentative lactic acid bacteria. Also Snell and Guirard (9) have reported isoleucine to be non-essential for *Streptococcus lactis* R. The leucine in their medium was supplied in the *dl* form. We² have found both leucine and isoleucine to be required by this organism³.

SUMMARY

1 Of seven samples of commercial *dl*-leucine tested, five showed appreciable isoleucine activity when tested by microbiological assay. Three samples were between 10 and 20 per cent as active as *dl*-isoleucine.

2 *dl*-Isoleucine was identified as a contaminant of one of these samples by isolation of this amino acid.

3 The inactivity of *d*(-)-leucine, tertiary *dl*-leucine, and *dl*-norleucine suggests that the total isoleucine activity is due to isoleucine or its optical isomers.

¹ The tosyl derivatives and melting point determinations were made by Dr Halvor N Christensen. We wish to express our appreciation for these and for his interest and helpful suggestions during this study.

² Hegsted, D M, and Wardwell, E D, unpublished data.

³ The culture of *Streptococcus lactis* R was kindly supplied by Dr E E Snell.

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STUDIES ON ANEMIA IN SWINE DUE TO PYRIDOXINE DEFICIENCY, TOGETHER WITH DATA ON PHENYL-HYDRAZINE ANEMIA*

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Nutritional microcytic anemia due to pyridoxine deficiency has been produced in the dog (1-4) and in the pig (5-7). The anemia fails to respond either to iron or to copper but is relieved by synthetic pyridoxine. Convulsions and ataxia have been shown by many authors (3-7) to accompany the deficiency. Peripheral nerve degeneration and hemosiderosis in the spleen, liver, and bone marrow have been reported by Wintrobe *et al* (7). The literature on pyridoxine deficiency in dogs and swine has recently been reviewed by the latter workers and they have reported in detail the manifestations of pyridoxine deficiency in swine.

Elevated plasma iron levels were first reported by Fouts *et al* (1) and later were recorded by McKibbin *et al* (4) and Wintrobe *et al* (7). A green pigment-producing substance in the urine of pyridoxine-deficient rats was reported by Lepkovsky and Nielsen (8). Later Fouts and Lepkovsky (9) found the substance in the urine of dogs under similar conditions, and Wintrobe *et al* (7) have reported its presence in the urine of pyridoxine-deficient pigs. Recently Lepkovsky, Roboz, and Haagen-Smit (10) have shown this substance to be xanthurenic acid, a metabolic product of tryptophane metabolism.

The purpose of this paper is to attempt to elucidate the mechanism by which anemia due to pyridoxine deficiency is produced by ascertaining the extent of blood destruction and by seeking the cause of the elevated serum iron.

Materials and Methods

Animals—Full details of the experimental methods have been given elsewhere (11). Pigs were obtained from the Bureau of Animal Industry, United States Department of Agriculture, Beltsville, Maryland. For this

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study twenty-seven animals were used. They were received at approximately 3 weeks of age and were divided into the following experimental groups: eleven pigs, pyridoxine deficiency, five pigs, iron deficiency, three pigs, combined pyridoxine and iron deficiency, one pig, hemolytic anemia (phenylhydrazine), seven pigs, inanition controls.

Diet—The animals were fed the basal diet consisting of Sheffield "new process" casein 26.1 per cent, sucrose 57.7 per cent, lard 11.0 per cent, salt mixture (Swine Salt Mixture 3 (11)) 5.2 per cent. In addition they received cod liver oil (Mead Johnson, 1800 units of vitamin A, 175 units of vitamin D per gm), 0.5 gm per kilo of body weight daily. Vitamins were supplied in crystalline form by placing them in capsules and administering them orally. The quantities of crystalline vitamins when these were given were as follows (mg per kilo of body weight daily): thiamine hydrochloride 0.25, riboflavin 0.12, nicotinic acid 1.20, pyridoxine hydrochloride 0.20, pantothenic acid 0.50, *p*-aminobenzoic acid 0.50, inositol 1.20, choline 10.0. In addition to the above diet the animals were given all eight vitamin supplements for 1 week. After this time the pyridoxine hydrochloride was omitted from the vitamin supplements given the pyridoxine-deficient group, iron pyrophosphate was omitted from the salt mixture given the iron-deficient group, and both the pyridoxine and iron salt were omitted from the diet of the combined pyridoxine- and iron-deficient group. The control animals were fed the same amount of diet as the pyridoxine-deficient group consumed, thus making them inanition controls. It should be noted that the iron deficiency in the animals given no iron pyrophosphate was not absolute, since the other salts fed no doubt contained small amounts of iron.

Bilirubin Determinations—These were made by the method of Malloy and Evelyn (12), with the Evelyn photoelectric colorimeter.

Urobilinogen Determinations—These were made by the method of Watson (13). The color developed by Ehrlich's reagent was measured on the Evelyn photoelectric colorimeter. Urine collections were made according to the method of Watson for 24 hour periods and the stools were collected continuously for 4 days.

Urinary Porphyrin Estimations—These were made by acidifying 100 ml of fresh urine with glacial acetic acid and then extracting with an equal volume of ethyl ether. This ether-soluble porphyrin fraction was then purified according to the method of Dobriner (14). Finally the total ether-soluble fraction in 5 per cent hydrochloric acid was placed in a fluorometer and the degree of fluorescence was noted and recorded in plus values ranging up to eight. If an increased amount of porphyrin was present, the protoporphyrin, deuteroporphyrin, and coproporphyrin fractions were separated according to Dobriner's procedure. Again the degree of fluores-

cence was noted. By this method even a slight increase beyond normal could be detected, although no attempt was made to measure the porphyrin output in absolute figures.

Serum Iron Determinations—These were made by precipitating 5 ml of serum with trichloroacetic acid at 90°. The filtrate was then adjusted to pH 6 with ammonium hydroxide and the iron determined with α, α -bipyridine after reduction with thioglycolic acid. A correction factor was added for the amount of iron which was carried down by the protein precipitate. Serum iron determinations by this method were accurate within ± 10 per cent. Great care was taken in cleaning the glassware and making the reagents free of iron.

Urinary Iron Determinations—The urine was digested with sulfuric acid and redistilled nitric acid. The iron content was then determined with α, α -bipyridine.

Xanthurenic Acid Excretion—This was determined qualitatively by neutralizing the urine to litmus, adding a few drops of ferric ammonium sulfate, and filtering. The depth of the green color was recorded by plus values ranging up to four.

Dialyzable Iron Determinations—15 ml of serum were obtained and 5 ml used for serum iron analysis. 10 ml were then pipetted into a cellophane bag equipped with a weighted sealed tube to increase the dialyzing surface. The serum was dialyzed at 37° against 10 ml of glass-redistilled water for 48 hours, the water changed, and the dialysis continued for 24 hours longer. The serum was then removed, the volume measured, and 5 ml used for analysis. The percentage which dialyzed was calculated and termed "dialyzable serum iron." An additional 5 ml from the dialyzing bag were placed in a second cellophane bag equipped with a weighted sealed tube and 5 ml of 0.2 N sulfuric acid were added. This was then dialyzed against 10 ml of 0.1 N sulfuric acid at 37° as before. The per cent which had then dialyzed was termed "acid-dialyzable serum iron." Controls were run on the glassware and reagents. All dialyzing bags and glassware were soaked in acid and rinsed many times with glass-redistilled water before use.

Studies on Blood Destruction—Measurements of blood destruction were made on pyridoxine-deficient pigs by determining urobilinogen excretion in the urine and stools, porphyrin excretion in the urine, and bilirubin and icterus index of the blood. It seemed desirable also to make similar measurements in a pig in which hemolytic anemia had been produced by giving phenylhydrazine, as well as in animals in which there was diminished hemoglobin formation as the result of iron deficiency.

The results obtained when a pig was given phenylhydrazine hydrochloride are summarized in Table I. The determinations of hemoglobin, volume

of packed red cells, reticulocytes, icterus index, serum bilirubin, and serum iron were made daily but in Table I they are averaged for 4 day periods. 4 day stool and 2 day urine collections were made and analyzed for urobilinogen but the results are expressed as mg per 24 hours. Urinary porphyrin collections were made on aliquots of 24 hour urine collections.

The pig excretes less urobilinogen in the stool and urine under normal conditions than might be expected. Furthermore, the normal serum bilirubin is extremely low and, as can be seen, does not rise to great heights, even while severe hemolysis is taking place. This is consistent with the

TABLE I
Phenylhydrazine Anemia

Period	Phenyl hydrazine	Hb	Hema- tocrit	Reticu- locytes	Icterus index	Bilru- bin	Serum iron	Urobilinogen per 24 hrs			Porphy- rinuria
								Urine	Stool	Total	
days	gm. per 4 days	gm. per cent	cc per cent	per cent		mg. per cent	per cent	mg	mg	mg	
1-4	0	11.2	38.3	1.6	2	0.3	173	0.1	0.4	0.4	0
5-8	0.5	10.7	36.3	1.7	4	0.1	284	0.1	1.4	1.4	
9-12	1.5	10.3	35.9	7.0	5	0.2	368	1.0	2.5	3.5	2+
13-16	3.0	10.9	33.5	10.6	6	0.6	354				
17-20	1.0	9.3	32.4	23.0	13	1.1	316	0.1	11.4	11.4	4+
21-24	3.0	8.2	30.7	31.2	10	1.3	295	1.0	39.8	40.8	
25-28	4.5	6.7	29.5	57.2	13	0.9	342	0.1	92.7	92.8	
29-32	4.0	6.6	27.8	45.6	15	1.8	352	1.6	27.1	28.7	
33-36	4.0	6.4	26.2	66.0	20	2.5	218	3.4	33.5	36.9	S+
37-40	4.0	5.5	30.7	50.8	27	2.7	175	1.0	16.6	17.6	S+
41-44	4.0	5.3	28.7	59.0	26	1.9	123	3.1	33.7	36.8	
45-48	4.0	5.6	24.4	37.2	27	3.4	127	8.5	24.8	33.3	
49-52	4.0	5.6	30.3	33.2	21	3.2	118	28.6	142.8	171.4	1+
53-56	4.0	6.3	30.5	34.1	25	2.3	166	15.2	45.5	60.7	3+
57-60	4.0	5.2	29.8	21.6	28	2.1	114	4.0	9.2	13.2	
Average					18	1.8		5.6	39.9	45.5	

statement of Lemberg (15) that the pig's serum contains only small quantities of bilirubin.

We have been unable to find an account of phenylhydrazine or hemolytic anemia in swine and for this reason report our data in some detail. The results indicated that as the hemolytic anemia developed a massive reticulocytosis occurred, the icterus index and serum bilirubin rose, and the urobilinogen and porphyrin excretion increased. This is in accord with observations on other species. The porphyrinuria was consistently due to an increase in the coproporphyrin fraction. The protoporphyrin and deuteroporphyrin fractions remained unchanged. As the blood destruction increased, the stools and urine became increasingly darker. It is of interest

that at the end of the experiment, in spite of the continued large doses of phenylhydrazine, the percentage of reticulocytes, the serum iron, and the urobilinogen and porphyrin excretion tended to diminish. This would seem to indicate a diminution in blood mass and consequently a diminution in the amount of hemoglobin which was available for hemolysis.

Table II presents a summary of the studies on blood destruction in pyridoxine anemia, phenylhydrazine anemia, iron deficiency anemia, and in control animals. The difference between the total urobilinogen excretion of the pyridoxine-deficient group, the control group, and the iron-

TABLE II
Studies on Blood Destruction

		Pyridoxine deficiency	Iron deficiency	Phenylhydrazine anemia	Inanition controls
No of animals		6	2	1	5
Hemoglobin terminal mean gm per cent		9.8	9.3	5.2	13.6
range		6.1-12.0	9.2-9.5		13.2-14.7
Icterus index	Mean	3	3	18	3
	Range	1-6	1-5	5-28	1-6
Reticulocytes, %	Mean	1	1	38	1
	Range	0.2-7.0	0.2-6.4	7.0-66.0	0.1-3.2
Porphyrinuria	Mean	0	0	5+	0
Serum bilirubin, total, mg %	"	0.18	0.55	1.87	0.48
	Range	0.04-0.45	0.54-0.56	0.2-3.4	0.08-1.09
Urobilinogen excretion, mg per 24 hrs					
Urine	Mean	0.1	0.1	5.6	0.1
	Range	0.0-0.2	0.0-0.2	0.1-28.6	0.0-0.2
Stool	Mean	1.3	1.6	39.9	0.6
	Range	0.1-3.6	1.3-2.0	2.5-142.8	0.2-1.3
Total	Mean	1.3	1.6	45.5	0.6
	Range	0.1-3.6	1.3-2.0	3.5-171.4	0.2-1.3

deficient group is not significant. The lower value for serum bilirubin in the pyridoxine-deficient animals as compared with the control and iron-deficient animals is also not significant. The averages given for the animal with phenylhydrazine anemia are calculated from the 9th through the 60th day.

It is clear that pyridoxine anemia does not correspond with a hemolytic anemia. There is no comparable reticulocytosis, elevation of icterus index or serum bilirubin, and no increased urobilinogen excretion. The only feature which phenylhydrazine anemia and pyridoxine anemia have in common is the increase in serum iron above normal.

Serum Iron Studies—Table III presents a summary of the serum iron determinations. The results are illustrated in Fig 1. In the control animals the serum iron values in most instances ranged between 90 and 180 γ per cent. The highest value obtained in a control animal was 218 γ per cent, and the lowest 71 γ per cent. In pyridoxine deficiency the increase in serum iron began at approximately the 4th week of the deficiency and reached its maximum (350 to 600 γ) between the 5th and 10th weeks. Following this the serum iron tended to fall off. In about half of the pyridoxine-deficient animals the serum iron fell to levels of 180 to 250 γ per

TABLE III
Summary of Serum Iron Determinations

	Control	Deficient in		
		Pyridoxine	Pyridoxine and iron	Iron
No. of animals	7	11	3	5
Duration of experiment, mean days	107	93	110	79
Duration of experiment, range days	32-151	6-119	74-172	28-122
No. of determinations of serum iron	85	93	31	51
Hemoglobin, terminal, gm %, mean*	14.0 \pm 0.45	8.9 \pm 0.89	3.9	9.0
Hemoglobin, terminal, range			3.8-4.0	7.4-9.7
Hematocrit, terminal, cc per 100 cc, mean*	41.3 \pm 1.67	29.3 \pm 2.51	15.2	29.8
Hematocrit, terminal, range			14.0-16.7	24.0-32.4
Mean corpuscular volume, cu μ , mean*	54.4 \pm 1.84	41.0 \pm 1.41	33.7	41.8
Mean corpuscular volume, range			30-38	38-47
Serum iron, γ %, mean*	142.7 \pm 5.32	373.6 \pm 14.31	52.4 \pm 3.23	48.0 \pm 2.31

* \pm the standard error of the mean

cent during the 2 weeks preceding death. In the iron-deficient animals the serum iron dropped to values of 30 to 75 γ per cent within 2 weeks and the values remained at this level throughout. In one animal pyridoxine deficiency was produced first and then iron deficiency was superimposed. At the end of 3 weeks of the pyridoxine-deficient régime, the serum iron had risen to 240 γ per cent. At this time the iron was removed from the diet. By the end of 30 days the serum iron had fallen to 59 γ per cent.

Combined pyridoxine and iron deficiency resulted in a greater anemia than either deficiency alone (see Table III). The volume of packed red

cells and hemoglobin was significantly lower. The combined deficiency also resulted in a lower mean corpuscular volume, which in one animal reached a low of 24 cu μ . The hemoglobin at this time was 2 gm per cent and the animal was so weak it was unable to stand for any length of time. The relatively mild anemia occurring in the iron-deficient animals (Table III) is not surprising, since these animals were only partially deficient in iron.

It seemed possible to us that the symptoms and pathology of pyridoxine deficiency might in part be due to iron toxicity but we were unable to detect any alteration in ataxia, convulsions, fatty liver, output of urinary pigment, or neurological lesions in the combined deficiency in comparison to deficiency of pyridoxine alone. It is to be noted, however, that the

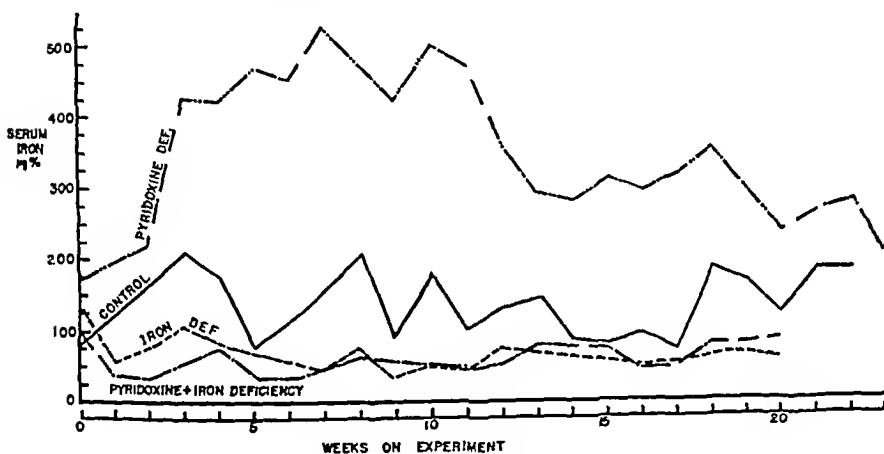


FIG 1 Serum iron values for a representative animal in each group

hemosiderosis we have described in pyridoxine deficiency (7) was entirely prevented in all three instances in which iron deficiency was associated with lack of pyridoxine.

Iron Excretion Studies—Urinary iron determinations were made on animals before and during the development of pyridoxine deficiency, and on control animals. The control animals excreted in the urine an average of 1.1 mg of iron per 24 hours, while the pyridoxine-deficient animals excreted an average of 1.0 mg per 24 hours before the deficiency developed and 0.9 mg in 24 hours during the deficiency. These differences are not significant. All animals excreted extremely small quantities of iron in the urine. From this it is obvious that urinary excretion of iron is of no practical significance. Even the above values are in all probability high, since collections entirely free from stool and cage contamination were impossible.

Iron balance studies were attempted. The results were extremely variable, but suggested that pyridoxine-deficient animals retain more iron than control animals. However, difficulties in analysis, stool collection, and exact measurement of dietary intake were so great that trustworthy results could not be obtained.

Dialyzable Serum Iron—Tompsett (16) has shown recently that the dialysis of plasma iron depends upon its state as ferrous or ferric iron. Ferrous iron is ultrafiltrable, whereas ferric iron is not. Acidification of plasma caused a reduction of iron to the ferrous state, with a consequent change from a non-ultrafiltrable to an ultrafiltrable state. For this reason dialysis studies were made on the serum of pyridoxine-deficient pigs to determine the state of the iron. The results are given in Table IV. Examination of the number of micrograms of iron which dialyzed against water in these animals, as compared with controls, reveals that approximately the same

TABLE IV
Dialysis of Serum Iron

Serum iron	Control (6 determinations) $M \pm SEM$	Pyridoxine deficiency (8 determinations) $M \pm SEM$	$M_1 - M_2$ σ difference
Initial, γ %	149.8 \pm 14.08	370.8 \pm 43.75	4.81
Dialyzable, %	24.7 \pm 1.78	12.1 \pm 2.12	4.51
" γ %	36.3 \pm 3.7	43.1 \pm 8.59	0.73
Acid-dialyzable, %	80.2 \pm 2.02	71.2 \pm 4.41	1.85
" γ %	119.0 \pm 10.41	259.1 \pm 30.14	4.39

* The difference between the means, divided by the standard deviation of the difference.

total amount dialyzed in both groups. This indicates that approximately 95 per cent of the increased serum iron in the serum of the pyridoxine-deficient animals is in a non-dialyzable form as ferric iron. The results on dialysis with acid, however, indicate that the greater proportion of this iron is reducible with acid. It must be noted, of course, that conclusions concerning the state of serum iron *in vivo* must not be drawn from these *in vitro* studies. Furthermore, it should be pointed out that the percentage of iron dialyzable following acidification depends upon the acid used and its concentration.

Urinary Porphyrin Studies—Because of the possibility that protoporphyrin was being synthesized normally, but not utilized, urinary porphyrin studies were made in all pyridoxine-deficient animals. It seemed logical that if iron could not be utilized and was stored in the tissues and carried in excess in the serum the same might be true of protoporphyrin. However, at no time was an increased excretion of urinary porphyrin found in

pyridoxine-deficient animals If porphyrins were formed normally, they were broken down and not excreted as such in the urine

Effect of Various Substances on Pyridoxine Anemia—If the formation of hemoglobin proceeds through pyrroles, porphyrin, and heme and if the anemia of pyridoxine deficiency is caused by a defect in the formation of one of these substances, it seemed possible that by supplying these substances or their precursors hemoglobin formation might be made to proceed at a normal rate and manner The results of these experiments are summarized in Table V The chlorophyll and sodium magnesium chlorophyllin were used as a source of porphyrin Purified liver extract was

TABLE V
Effect of Various Substances on Pyridoxine Anemia

Pig No	Therapy			Hb change	Hemato-crit change	Reticu- loocytes	
	Substance	Dose per day	Duration				
			days	gm per cent	cc per cent	per cent	
7-80	Chlorophyll, gm	0.4	Subcutaneous	10	0	-6.0	6.4
7-84	Na Mg chlorophyllin, gm	1.0	Oral	8	-1.7	+6.9	2.4
7-80	Liver extract, units	10	Intramuscular	10	0	-1.8	0.8
7-82	" " "	10	"	8	+0.6	-3.4	4.2
7-78	Tryptophane, gm	0.5	Oral	10	-0.2	+2.0	0.8
7-80	" " "	0.5	"	14	-0.6	-4.0	11.4
7-79	Corn oil, gm	50	"	45	-4.3	-12.4	3.0
7-83	" " "	50	"	9	+0.8	-2.0	1.8
7-78	Crude Hb, gm	100	"	13	-0.4	-0.6	0.4
7-78	Hemin, gm	1.0	"	10	+1.6	-0.2	0.8
7-80	Iron ascorbate, mg	50	Intravenous	20	+3.1	+8.0	12.4
7-78	" " "	50	"	10	-3.7	-14.6	1.2
7-79	" " "	50	"	20	-1.1	-3.0	2.6
7-78	" " "	50	"	7	+0.2	+1.4	0.6

given to two animals to ascertain whether the anti-pernicious anemia factor would be effective in relieving this anemia There was no significant effect Because of the relationship between pyridoxine deficiency and tryptophane metabolism, as shown by Lepkovsky *et al* (10), and because of the possibility that tryptophane is concerned with porphyrin synthesis, this substance was tried In the first animal (Pig 7-78) no response was obtained In the second animal (Pig 7-80) a reticulocyte increase of 11.8 per cent occurred However, because of an absence of hemoglobin and hematocrit response, it was concluded that this reticulocytosis was not significant It was noticed that the urinary excretion of xanthurenic acid in these animals was greatly enhanced This confirms the finding of Lepkovsky and co-workers (10)

The synergistic action between unsaturated fatty acids and pyridoxine in the alleviation of acrodynia in the rat, produced by a diet inadequate in the essential unsaturated fatty acids, has been described by Quackenbush *et al* (17). Therefore, it seemed desirable to determine whether these fatty acids would affect pyridoxine anemia. Their ineffectiveness in this respect can be seen in Table V (corn oil).

The possibility occurred that, although there is marked storage of iron in the tissues of pyridoxine-deficient animals and although the serum iron is elevated, the iron might in some way be in a non-utilizable form. To test this, iron ascorbate was given intravenously to Pig 7-80. A definite response seemed to occur and at the same time the hemoglobin and hematocrit rose. On the 9th day the reticulocytes reached 12.4 per cent and remained at this level for 3 days. However, since the animal died at the end of this experiment, the value of this observation seemed questionable. Iron ascorbate was then given to two additional animals, in one animal twice. In all three instances there was no effect. Therefore, it was concluded that the response in Pig 7-80 was caused by some other factor and was not due to the iron given intravenously.

DISCUSSION

The theoretical factors which could cause a rise in serum iron are six, namely, increase in the absorption of iron, decrease in the excretion of iron, decrease in utilization for hemoglobin synthesis, increase in the iron liberated from hemoglobin by hemolysis, increase in iron liberated from the tissues, and diminished uptake of iron by the tissues. We have shown that in pyridoxine deficiency iron in the tissues is abundant. It would seem then that the iron is going from the serum into the tissues at an increased rate and, therefore, it is unlikely that the ferremia could be due either to increase in iron liberated from the tissues or to diminution of the iron taken up by the tissues. That there is not an increased rate of hemolysis can be seen from Table II. Therefore, the ferremia must be due either to a continued or increased retention of iron, either through increased absorption or decreased excretion in the intestines, or is due to diminished hemoglobin formation, or a combination of both.

A diminution in hemoglobin formation occurs, since anemia develops without evidence of hemolysis. That this factor alone is not great enough to cause a ferremia is indicated by the fact that in combined pyridoxine and iron deficiency the ferremia and hemosiderosis do not occur, and that in anemia due to protein deficiency,¹ which is equally severe, ferremia and hemosiderosis of the tissues are not present. It would seem that the ferremia of pyridoxine deficiency is caused by continued retention of iron at a

¹ Wintrobe, M. M., and Cartwright, G. E., unpublished data.

time when its utilization for hemoglobin formation is at a minimum. Whether iron is retained normally or whether the retention is increased in pyridoxine deficiency, we are unable to state. The possibility that the retention is increased in pyridoxine anemia is interesting, since this would be a striking exception to the finding of Hahn and Whipple (18) in dogs and in humans (19), namely, that the absorption of iron is dependent on the need of the body for iron. In such conditions as pernicious anemia and hemochromatosis, in which the iron stores are abundant, these workers (19) found that there is very little absorption of iron. In pyridoxine deficiency tissue iron is abundant, its utilization is diminished, and yet the body continues to absorb iron.

Since combined pyridoxine and iron deficiency prevents ferremia, it might be expected that there would be no ferremia in natural pyridoxine deficiency in humans, if such exists. For, if the diet were so poor as to be deficient in pyridoxine, it would also be likely to be deficient in iron. This is in keeping with our findings² and with the findings of Moore *et al* (20) that vitamin-depleted patients have low rather than high serum iron levels.

Pyridoxine anemia is similar to pernicious anemia in several respects. In both conditions there is an increase in serum iron, hemosiderosis of the tissues, hyperplastic bone marrow, and neurological lesions. That they are not the same is evidenced by a microcytosis in one and a macrocytosis in the other. Also, pyridoxine anemia does not respond to liver extract and pernicious anemia² does not respond to pyridoxine. However, since they are similar in several aspects, knowledge of the mechanism of pyridoxine anemia might aid in elucidating the mechanism of pernicious anemia.

SUMMARY

1 The catabolism of hemoglobin in the pig proceeds in a manner similar to that in man.

2 Studies on serum bilirubin, per cent of reticulocytes in the blood, icterus index, urobilinogen excretion in the stool and urine, urinary excretion of porphyrin, and comparison with anemia induced by phenylhydrazine, indicate that an increased rate of hemolysis does not occur in pyridoxine deficiency.

3 By restricting the dietary intake of iron in pyridoxine-deficient animals, the hemosiderosis of the tissues has been prevented, and the ferremia has not only been prevented but the serum iron has been maintained at the low level seen in iron deficiency.

4 The ataxia, convulsions, neurological lesions, and fatty livers of pyridoxine deficiency were not altered by limiting the iron intake in the combined deficiency.

² Cartwright, G. E., and Wintrobe, M. M., unpublished data.

5 The urinary excretion of iron in pyridoxine deficiency is insignificant and is not altered from the normal

6 The increased iron in the serum of pyridoxine-deficient animals is in the ferric state

7 Chlorophyll, sodium magnesium chlorophyllin, anti-pernicious anemia liver extract, tryptophane, corn oil, crude hemoglobin, hemin, and iron ascorbate were ineffective in stimulating blood formation in pyridoxine-deficient animals under the conditions of this experiment

8 Evidence is presented that the ferremia and hemosiderosis are due to the continued absorption or decreased excretion of iron at a time when its utilization for hemoglobin formation is at a minimum and when the body tissues are abundant with iron. This is an exception to the observation that in other types of anemia the absorption of iron appears to be dependent upon the need of the body for iron

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THE STRUCTURAL SPECIFICITY OF CHOLINE FOR THE GROWTH OF TYPE III PNEUMOCOCCUS*

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Rane and Subbaw (1) have shown that choline is required for the growth of pneumococci. In preliminary experiments designed to develop a microbiological assay for choline it was found that ethanolamine would support the growth of a strain of Type III pneumococcus in the absence of choline. Other compounds related to choline and ethanolamine were then tested for their activity in promoting growth of this organism. The compounds studied were chosen to investigate the structural specificity of choline for the growth of the pneumococcus as well as the manner in which it and the related compounds act. It was found that the active compounds contained $\text{N}-\text{C}-\text{C}-\text{OH}$ or $\text{N}-\text{C}-\text{C}-\text{C}-\text{OH}$ linkages. Substitution of or through the hydroxyl group resulted in complete inactivation of the molecule. Ethanolamine was the only naturally occurring substance which supported growth in the absence of choline. However, 10 times as much ethanolamine as choline on a molecular basis was required to produce maximum growth.

EXPERIMENTAL

Organism—A CHA strain of Type III pneumococcus obtained from Dr. L. H. Schmidt at the Christ Hospital Institute for Medical Research, Cincinnati, was used throughout this work. The stock culture was passed through mice three times a week and the heart blood cultured in veal-phosphate broth. The inoculum was prepared by subculturing 0.1 ml. of the stock culture into veal-phosphate broth enriched with 0.1 per cent glucose and incubating 6 hours. This young culture was centrifuged, washed, and resuspended in an equal volume of the sterile basal medium. 0.1 ml. of this suspension was used to inoculate 10 ml. of the medium.

Medium—A semisynthetic medium developed in this laboratory supported luxuriant growth of this organism.¹ The constituents of the medium without choline are given in Table I. Maximum growth of the Type

* The data are taken from the dissertation submitted to the Faculty of the Graduate School, University of Cincinnati, June 1943, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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¹ Details of this medium will appear in a separate paper.

III pneumococcus was obtained in this semisynthetic medium with the addition of 5 γ of choline chloride per ml of medium. The addition of choline chloride of from 0.1 to 5 γ per ml resulted in a corresponding increase in growth as measured turbidimetrically. No measurable growth occurred when the choline was omitted. Stock solutions of the vitamins, except ascorbic acid, were made up separately in such concentrations that 0.1 ml of each was sufficient for 10 ml of medium. The ascorbic acid was weighed and added to the medium just before autoclaving.

TABLE I
Semisynthetic Medium for Type III Pneumococcus

Basal medium*	
Vitamin free casein hydrolysate, gm N	0.5
KH ₂ PO ₄ , gm	5.0
MgSO ₄ 7H ₂ O, gm	0.4
Cystine, mg	25.0
Distilled water to make 800 ml	
Added to 8 ml portions of basal medium	
Calcium pantothenate, γ	10.0
Nicotinic acid, γ	10.0
Biotin (crystalline), γ	0.01
Thiamine, γ	20.0
Creatine, γ	50.0
Ascorbic acid, mg	3.0
Dextrose,† mg	50.0

* The pH of the basal medium was adjusted to 7.8, the mixture was heated to boiling, filtered while hot, and stored in the refrigerator at least 2 days before use.

† Added to the medium after autoclaving.

Procedure

The growth tests were carried out in Pyrex test-tubes which had been matched in the turbidimeter. The compound to be tested was added to these tubes in molecular concentrations equivalent to 5 γ of choline chloride per ml of medium. The medium was then added, the volume brought up to 10 ml with distilled water, and the tubes autoclaved for 15 minutes at 10 pounds pressure. The sterile glucose was added aseptically and the tubes inoculated. If growth resulted, the experiment was repeated until the concentration at which maximum growth occurred was determined. If no growth resulted from the addition of the compound, a wider range of concentrations was tried.

The compounds in concentrations equal to 5 γ of choline per ml were also added to tubes containing suboptimal and optimal concentrations of choline to test the additive as well as any stimulating or inhibiting effects.

None of the compounds tested inhibited or accelerated growth in the presence of optimal concentrations of choline

Measurement of Growth—Growth was measured turbidimetrically in a comparator described by Kiebs *et al* (2) The turbidity which developed in tubes containing the various compounds was compared after 12 hours incubation with that produced in tubes containing 0, 1, 3, and 5 γ of choline chloride per ml Where there was slight or no growth, the cultures were allowed to incubate at least 36 hours to allow time for slow utilization to become apparent Since the amount of activity shown within 12 hours was unaltered qualitatively or quantitatively on further incubation, only the results of the 12 hour incubation period are given here

Source of Compounds—We are indebted to Dr Halvor Christensen for the calcium salt of the phosphorylcholine chloride, to Dr V du Vigneaud for the diethylmethyl- β,γ -dihydroxypropylammonium chloride, α,α -dimethylcholine chloride, triethylcholine chloride, and dimethylethylhydroxyethylammonium chloride, to the Carbide and Carbon Chemicals Company for the tetraethanolammonium hydroxide, α -ethyl- α -hydroxymethylethanolamine, N-phenylethanolamine, diethylethanolamine, dimethylethanolamine, methyldiethanolamine, and diethanolamine The ethanolamine, N-acetyethanolamine, β -methoxyethylamine, triethanolamine, and γ -diethylaminopropanol were obtained from the Eastman Kodak Company, α -methyl- α -hydroxymethylethanolamine, α,α -dihydroxymethylethanolamine, and α,α -dimethylethanolamine from the Commercial Solvents Corporation, acetylcholine chloride, acetyl- β -methylcholine chloride, carbamylcholine chloride, urethane of β -methylcholine chloride, and glycine from Merck and Company, Inc

Results

The results have been summarized in Tables II to IV, where the compounds tested have been arranged according to the atom of the ethanolamine moiety on which substitution occurs The concentration producing maximum turbidity is given both in micrograms per ml of medium and in choline equivalents The turbidity obtained with this most effective concentration is expressed as percentage of the turbidity obtained with 5 γ of choline chloride

Substitution on Nitrogen Atom—An analysis of the relative activities of the compounds obtained by substitution on the nitrogen atom of the ethanolamine moiety indicates that the trimethyl groups of the nitrogen atom in the choline molecule are not the essential part Replacement of any one or all of the trimethyl groups with ethyl groups does not alter the ability of the molecule to produce growth Whether the nitrogen is primary, secondary, tertiary, or quaternary seems to have no relation to the

growth activity. The greater activity of the di- and triethanolamine over the monoethanolamine and tetraethanolammonium hydroxide is difficult to explain. Ethanolamine from two different sources was tested, as well as some repurified by crystallization of the ovalate. The results confirmed those given and thus eliminate the factor of possible impurity. On a

TABLE II

Activity of Choline Derivatives on Growth of Type III Pneumococcus. Substitution on Nitrogen Atom

Compound	Structural formula	Most effective concentration	Choline chloride equivalents	Activity
I Choline chloride	$(CH_2)_3N(Cl)-CH_2-CH_2-OH$	5.0	5	100
II Dimethylethanolamine	$(CH_3)_2N-CH_2-CH_2-OH$	3.2	5	100
III Methyl-diethanolamine	$CH_3-N(CH_2-CH_2-OH)_2$	4.2	5	100
IV Triethylcholine chloride	$(C_2H_5)_3N(Cl)-CH_2-CH_2-OH$	6.0	5	100
V Diethylethanolamine	$(C_2H_5)_2N-CH_2-CH_2-OH$	4.2	5	100
VI Dimethylethylhydroxyethylammonium chloride	$(CH_3)_2(C_2H_5)N(Cl)CH_2-CH_2-OH$	5.5	5	100
VII Ethanolamine	$NH_2-CH_2-CH_2-OH$	22	50	80
VIII Diethanolamine	$NH(CH_2-CH_2-OH)_2$	7.5	10	95
IX Triethanolamine	$N(CH_2-CH_2-OH)_3$	6.4	5	95
X Tetraethanolammonium hydroxide	$HO-N(CH_2-CH_2-OH)_4$	30	20	85
XI N-Acetyethanolamine†	$CH_3-CO-NH-CH_2-CH_2-OH$	32	50	23
XII Phenylethanolamine	$C_6H_5-NH-CH_2-CH_2-OH$			0‡

* Per cent of turbidity obtained with 5 γ of choline chloride per ml of medium

† Added after autoclaving

‡ No growth was obtained over a range of 1 to 50 γ per ml of medium

molecular basis, approximately 10 times as much ethanolamine as choline is required to produce maximum growth. The slight activity of N-acetyethanolamine is probably due to ethanolamine formed by hydrolysis of the acetyl group in the slightly alkaline medium. Substitution of a phenyl group on the nitrogen results in a completely inactive compound.

Substitution on α-Carbon Atom—Substitution on the α-carbon atom pro-

TABLE III

Activity of Choline Derivatives on Growth of Type III Pneumococcus Substitution on α Carbon Atom

Compound	Structural formula	Most effective concentration	Choline chloride equivalents	Activity
		γ per ml	γ per ml	per cent
I Choline chloride	$(\text{CH}_3)_3\text{N}(\text{Cl})-\text{CH}-\text{CH}-\text{OH}$	5.0	5	100
XIII α Ethylethanolamine	$\text{NH}-\text{CH}-\text{CH}_2-\text{OH}$ C_2H_5	5.1	7.5	80
XIV 2 Nitro 1 butanol	$\text{NO}_2-\text{CH}-\text{CH}_2-\text{OH}$ C_2H_5			0†
XV α Ethyl α hydroxymethylethanolamine	$\text{NH}-\text{C}-\text{CH}-\text{OH}$ $\text{CH}-\text{OH}$ C_2H_5	16	20	100
XVI α Methyl α hydroxymethylethanolamine	$\text{NH}-\text{C}-\text{CH}-\text{OH}$ $\text{CH}-\text{OH}$ CH_3	28	40	90
XVII α, α -Dihydroxymethylethanolamine	$\text{NH}_2-\text{C}-\text{CH}-\text{OH}$ $\text{CH}-\text{OH}$ CH_3	40	50	5
XVIII α, α -Dimethylethanolamine	$\text{NH}-\text{C}-\text{CH}-\text{OH}$ CH_3 CH_3	37	50	31
XIX α, α -Dimethylcholine chloride	$(\text{CH}_3)_3\text{N}(\text{Cl})-\text{C}-\text{CH}-\text{OH}$ CH_3	6.0	5	20
XX Serine	$\text{NH}-\text{CH}-\text{CH}-\text{OH}$ COOH			0†

* Per cent of turbidity obtained with 5 γ of choline chloride per ml of medium

† No growth was obtained over a range of 1 to 50 γ per ml of medium

duces compounds of varied activity. The substitution of an ethyl group produces a compound (No. XIII, Table III) almost as active as choline

TABLE II
Activity of Choline Derivatives on Growth of Type III Pneumococcus Substitution on
 β Carbon Atom

Compound	Structural formula	Most effective concentration	Choline chloride equivalent	Activity
		γ per ml	γ per ml	per cent*
I Choline chloride	$(CH_3)_3N(Cl)-CH-CH_2-OH$	5.0	5	100
XXI Ethylamine	$NH_2-CH_2-CH_3$			0†
XXII Ethylenediamine	$NH_2-CH_2-CH_2-NH_2$			0†
XXIII Glycine	NH_2-CH_2-COOH			0†
XXIV Sarcosine	$CH_3-NH-CH_2-COOH$			0†
XXV Betaine	$(CH_3)_3N-CH_2-CO$ O			0†
XXVI β-Methoxyethylamine	$NH_2-CH_2-CH_2-O-CH_3$			0†
XXVII Urethane of β-methylcholine chloride†	$(CH_3)_3N-CH_2-CH_2-O-CO$ Cl CH ₃ CH ₃			0†
XXVIII Carbamylcholine chloride†	$(CH_3)_3N-CH_2-CH_2-O-CO-NH_2$ (Cl)			0†
XXIX Acetylcholine chloride†	$(CH_3)_3N-CH_2-CH_2-O-CO-CH_3$ (Cl)		5	7
XXX Acetyl β-methylcholine chloride†	$(CH_3)_3N(Cl)-CH_2-CH_2-O-CO$ CH ₃ CH ₃		5	7
XXXI Phosphorylcholine chloride (Ca)	$(CH_3)_3N(Cl)-CH_2-CH_2-O-PO_3Ca$			0†
XXXII γ-Diethylaminopropanol	$(C_2H_5)_2N-CH_2-CH_2-CH_2-OH$	5.8	6	100
XXXIII β, γ-Propriolol α-diethylamine	$(C_2H_5)_2N-CH_2-CH(OH)-CH_2-OH$	10.0	10	100
XXXIV Diethylmethyl β, γ-dihydroxypropylammonium chloride	$(C_2H_5)_2N-CH_2-CH(OH)-CH_2-CH_2-OH$ Cl OH OH	7.0	5	20
XXXV Carnitine	$(CH_3)_3N(OH)-CH_2-CH_2-CH_2-COOH$ OH COOH			0†

* Per cent of turbidity obtained with 5 γ of choline chloride per ml of medium

† No growth was obtained
† Added after autoclaving

However, when the remaining hydrogen is replaced with a methyl alcohol group (Compound XV, Table III) the concentration must be increased 4 times to produce equivalent growth. When the ethyl group is replaced with a methyl group (Compound XVI, Table III), the concentration must be increased 8 times to obtain maximum growth. A further decrease in activity results from the substitution of two methyl or two hydroxymethyl groups on this carbon atom (Compounds XVII, XVIII, and XIX of Table III). The carboxyl group in serine makes this compound inactive.

Substitution on β -Carbon Atom—The relative activities of the compounds in which the β -carbon atom is substituted emphasize the structural specificity of choline and ethanolamine. This pneumococcus cannot synthesize the needed substance by oxidizing ethylamine or reducing glycine or sarcosine. Neither can the organism hydrolyze the ester or ether linkages through the hydroxyl group. The activity of the acetyl compounds is probably due to their hydrolysis to choline in the slightly alkaline medium. The high activity of the two homologues (Compounds XXXII and XXXIII, Table IV) is interesting as well as surprising. The difference in the activities of the two dihydroxy compounds (Nos XXXIII and XXXIV, Table IV) is unaccountable, unless it could be due to differences of configuration of the asymmetric carbon atom.

DISCUSSION

From the results given above it is seen that this pneumococcus requires a molecule containing a $\text{N}-\text{C}-\text{C}-\text{OH}$ or $\text{N}-\text{C}-\text{C}-\text{C}-\text{OH}$ linkage. Substitution of ethyl or methyl groups on the nitrogen or α -carbon atoms increases the activity, except in the case of the α,α -dimethyl and dihydroxymethyl derivatives, in which case the activity is decreased. The addition of a phenyl or acetyl group to the nitrogen atom or a carboxyl group to either carbon atom inactivates the molecule. It is necessary that the hydroxyl group remain free, since any substitution of or through this group results in loss of activity.

The inactivity of ethylamine, glycine, sarcosine, carnitine, and serine precludes the suggestion that choline or ethanolamine is required as a precursor of these substances.

From this study several conclusions may be drawn as to the mode of action of choline and ethanolamine in the nutrition of this pneumococcus. Experimental evidence of the action of choline in animal metabolism suggests at least three different functions: (1) as a source of labile methyl groups, (2) as a precursor of acetylcholine, and (3) as an essential constituent of the phospholipids. The activity of triethylcholine, diethylethanolamine, and similar compounds plus the inactivity of methionine, betaine, phosphorylcholine, and other compounds containing labile methyl groups provides evidence that the rôle of choline in the pneumococcal metabolism

is not that of transmethylation. The inactivity of acetylcholine indicates also that choline is not the precursor of acetylcholine.

The fact that ethanolamine can replace choline suggests the formation of phospholipids as the most likely explanation of the action of choline in pneumococcal metabolism. It has not yet been demonstrated that the pneumococcal cell contains phospholipids, but it has been shown that phospholipids are present in other bacterial cells (3, 4).

Ethanolamine may be converted by the organism to choline or perhaps the two compounds function independently to form two phospholipids such as lecithin and cephalin. The fact that ethanolamine is required in 10 times the concentration for equivalent growth does not offer evidence for either mode of action, except to indicate that choline is probably not demethylated to form ethanolamine. It has been observed that in the ethanolamine cultures the organisms consistently grew in long chains, while no chains were found in cultures containing choline or choline plus ethanolamine. That substitution of ethanolamine for choline does not alter the cellular metabolism to any great degree was demonstrated by the maintenance of virulence after twenty subcultures in the semisynthetic medium containing ethanolamine in place of choline.

If choline and ethanolamine go to form phospholipids in the pneumococcal cell similar to those found in animal cells, it is rather surprising that phosphorylcholine is inactive. However, the phospholipid synthesis may require a free hydroxyl group on the choline or ethanolamine molecule.

There is no correlation between the activity of the various choline derivatives and related compounds in promoting growth of pneumococci and their action in the prevention of perosis and promotion of growth of chicks, the promotion of growth of rats on a diet containing homocystine but lacking in methionine, and the prevention of fatty livers and hemorrhagic kidneys in rats, as summarized by Moyer and du Vigneaud (5). It is interesting to note that only in the pneumococcal nutrition has ethanolamine been shown to replace choline.

It is quite possible that the function of choline and ethanolamine in pneumococcal metabolism is entirely different from any of the known roles in mammalian metabolism. This fact alone makes the continued study of choline metabolism of the pneumococcus of interest.

SUMMARY

Some thirty-five different compounds related to choline have been tested for their growth-promoting activity for a Type III pneumococcus, grown in a semisynthetic medium. It was found that active compounds contained a $\text{N}-\text{C}-\text{C}-\text{OH}$ or $\text{N}-\text{C}-\text{C}-\text{C}-\text{OH}$ linkage. Substitution of or through the hydroxyl group resulted in complete inactivation of the

molecule Of all the naturally occurring compounds tested, only ethanolamine was able to support the growth of this pneumococcus in the absence of choline On a molecular basis ethanolamine was required in 10 times the concentration of choline for maximum growth The possible role of choline in pneumococcal metabolism has been discussed

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THE DETERMINATION OF *p*-AMINOBENZOIC ACID BY ASSAY WITH *CLOSTRIDIUM ACETOBUTYLICUM**

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Since the demonstration of the antagonistic action of *p* aminobenzoic acid (PAB) toward the activity of the sulfonamides and of its requirement by several species of microorganisms, there is much interest in methods for the determination of this substance. The available chemical methods do not possess sufficient sensitivity for use on natural materials. Landy and Dicken (1) have described a microbiological assay using *Acetobacter suboxydans* as the test organism. Also Lewis (2) has presented a method in which *Lactobacillus arabinosus* 17-5 is employed. Rubbo *et al* (3), Park and Wood (4), and McIlwain (5) have reported the use of *Clostridium acetobutylicum* in a semiquantitative manner. Thompson *et al* (6) have developed an assay with an 'aminobenzoicless' mutant of *Neurospora crassa* developed by Tatum and Beadle (7).

In this paper we will describe the determination of PAB by the growth response of *Clostridium acetobutylicum* Strain S9. The chief advantage of this method is the short incubation time, 20 to 24 hours, required. The error is of the order usual in microbiological assay, *i e*, ± 10 per cent.

EXPERIMENTAL

Culture—*Clostridium acetobutylicum* Strain S9 is used as the test organism. This strain will attain maximum growth on a basal medium of glucose, ammonium phosphate (dibasic), salts, biotin, and PAB (8). However, 3 days are required for maximum growth on this lean medium. An effort was made to reduce the growth period by the use of suitable supplements.

The optimum concentration of phosphate ion and of NH_3 was 0.05 to 0.10 per cent. Addition of a reducing agent shortened the lag phase, sodium hydrosulfite was the best. The addition of 5 mg per 10 cc of a "PAB-free" casein hydrolysate (described below) plus cystine and tryptophane reduced the time required for maximum growth to 24 hours. This supplement could be replaced by a mixture of eighteen amino acids. On the casein hydrolysate-basal medium, concentrations of Na_2SO_4 or NaCl up to 20 mg per cc had no inhibitory effects.

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The stock culture is carried on sterile soil and each week a loopful is inoculated into a medium consisting of glucose, ammonium phosphate (dibasic), ammonium acetate and salts (all at one half the concentrations given in Table I), and 0.5 per cent peptone. After 24 to 30 hours incubation, 4 drops of this culture are added to a tube containing 5 cc of the basal medium, 0.005 γ of PAB, and water to make 10 cc. When 18 to 24 hours old, 1 drop is added to each tube of the assay. For assays on successive days, inoculum may be prepared by inoculating another tube of the stock medium. The first to fourth transfers on this medium can be used. More transfers on the stock medium may result in a weakened culture and hence it is best to start again from the soil spore stock. Centrifuging and resuspending the cells, even when freshly sterilized basal medium was used as

TABLE I
Composition of Basal Medium

Glucose, gm per 100 cc	4.0
Ammonium phosphate (dibasic), gm per 100 cc	0.2
Ammonium acetate, gm per 100 cc	0.4
Salt mixture,* gm per 100 cc	0.2
"PAB-free" casein hydrolysate, cc per 100 cc	1.0 (100 mg)
Cystine, mg per 100 cc	20
Tryptophane, mg per 100 cc	15
Biotin, γ per 100 cc	0.2
Na ₂ S ₂ O ₄ · 2H ₂ O, mg per 100 cc	10
Reduced iron, mg per tube	5

* Salt mixture: K₂HPO₄, 10 gm; K₂H₂PO₄, 10 gm; MgSO₄ · 7H₂O, 4.0 gm; NaCl, 0.2 gm; MnSO₄ · 4H₂O, 0.2 gm; FeSO₄ · 7H₂O, 0.2 gm.

the suspending fluid, caused an increased lag in growth. By bringing up the inoculum on the synthetic stock medium it is possible to avoid this centrifugation, as the amount of PAB added in 1 drop is negligible (about 0.03 millimicrogram).

Basal Medium.—The constituents of the basal medium are given in Table I. A stock solution is prepared containing the ammonium phosphate (dibasic), ammonium acetate, salt mixture, casein hydrolysate, cystine, and tryptophane. The cystine is previously dissolved with the addition of a minimum amount of HCl. This solution is slightly turbid, but it is unnecessary to filter it. A stock solution of biotin (crystalline material, S. M. A. Corporation) is prepared containing 0.25 γ of biotin per cc. Both stock solutions are stored in the refrigerator with added toluene and chloroform. In the preparation of 1 liter, for example, of the basal medium, 40 gm of glucose and 8.0 cc of biotin solution are added to 500 cc of the salts-casein hydrolysate mixture, the solution is adjusted to pH 6.7 \pm 0.1, and

diluted to 1 liter Just before use, 100 mg of sodium hydrosulfite are added

PAB-Free Casein Hydrolysate—100 gm of Labco "vitamin-free" casein are refluxed 20 hours with 1 liter of 20 per cent HCl The hydrolysate is reduced *in vacuo* to a thin syrup, 300 cc of water added, and the water again removed This is repeated twice, the resulting syrup dissolved in water, brought to pH 4 with NaOH, and diluted to 1 liter

100 cc of this hydrolysate are stirred with 2 gm of norit for 30 minutes on a steam bath, then cooled, and filtered While the norit removes much of the PAB, the filtrate is not sufficiently low in this factor to permit its use in the basal medium The solution is brought to pH 2 with H_2SO_4 and continuously extracted with ether for 24 hours Longer extraction removes so much of the stimulatory amino acids that maximum growth is not attained in 24 hours The residue is brought back to 100 cc, thus 1 cc is equivalent to about 100 mg of casein This extracted material is suitable for use and is stored at pH 2 in the refrigerator

Procedure

Bacteriological test-tubes (19 × 150 mm) are used for the assay Approximately 5 mg of reduced iron are first added to each tube Appropriate aliquots of the standard PAB solution and of the materials to be assayed are placed in the tubes, and water is added to bring the volume to 5 cc The basal medium is made up just before use and 5 cc are added to each tube The tubes are plugged with cotton and autoclaved 15 minutes at 15 pounds pressure

After autoclaving, the tubes are cooled and inoculated as described earlier An anaerobic jar is prepared by filling a suitable container one-fourth full of oats and adding just enough water to cover the oats The assay tubes are placed in the jar and a glass top is sealed on with plasticine The respiration of the oats removes the oxygen and establishes a partial pressure of CO_2 The jar is placed in the incubator at 37° for 20 to 24 hours

After incubation the tubes are shaken well, their contents are transferred to Evelyn tubes, and the turbidity is read in an Evelyn photoelectric colorimeter with a 660 $m\mu$ filter against the uninoculated basal medium set at 100 PAB values may then be read from the standard curve

Standard Curve—The assay range is approximately 0.3 to 1.5 millimicrograms of PAB per tube A standard solution containing 0.5 γ of PAB per cc is diluted to 0.5 millimicrogram per cc Aliquots of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 5.0 cc are used, and a standard curve (Fig. 1) based on turbidities resulting from growth with these additions is drawn A fresh standard solution of PAB is prepared each month

A reference dry material is run with each assay as an additional check of

the standard and of the completeness of the medium. A Difco yeast extract has been used and assays 80 γ of PAB per gm. Any assay in which the PAB content of this preparation is not within ± 10 per cent of this value is discarded. This material is stored in a desiccator and a fresh solution is made up monthly, preserved with toluene and chloroform, and kept in the refrigerator. The reference material serves as a check on the potency of the culture and on the PAB standard.

Great care must be taken to keep all of the constituents of the medium free of PAB. All glassware should be cleaned with dichromate cleaning solution. Also the handling of PAB in the laboratory should be kept to a minimum. After the preparation of a series of PAB derivatives our laboratory became contaminated with it. The culture apparently no longer

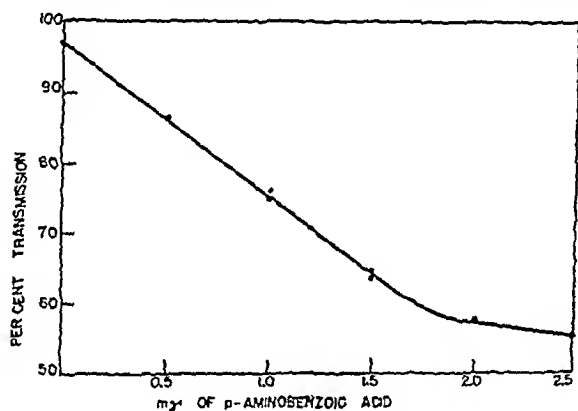


FIG 1 Response of *Clostridium acetobutylicum* to added *p* aminobenzoic acid

needed PAB, and only after repeated cleaning of the laboratory and equipment could the assay be made to work satisfactorily. Most samples of cystine contain PAB and should be checked before use.

Specificity of Assay—The activity of compounds related to PAB for the test strain has been reported (9). *p*-Nitrobenzoic acid has equal molar activity, and procaine about 10 to 20 per cent activity. *p*-Aminophenyl acetic acid has a maximum activity of 0.002 per cent. The methyl and ethyl esters and acetyl and benzoyl derivatives show only traces of activity.

Freeing of PAB from Natural Materials—Many investigators have reported the occurrence of sulfanilamide inhibitor (PAB or its equivalent) in a bound form in various natural materials. Acetylation (10) or other conjugation (11, 12) of PAB are known to occur on feeding or injecting animals. Blanchard (13) found, on autolysis of bakers' yeast, increases in the PAB which could be isolated and in the total diazotizable amines

present Lewis (2) observed increased PAB values after autoclaving with 1 N NaOH McIlwain (5), using *Clostridium acetobutylicum*, obtained maximum plasma PAB values on autoclaving with 1 N HCl 6 N H₂SO₄ for 1 hour at 15 pounds pressure gave optimum release of PAB when the *Neurospora* assay was used (14)

We have studied the liberation of PAB from liver by autolysis, enzyme hydrolysis, acid, and alkali For all of the experiments a dried powdered liver sample (Liver A) was used

Data on the liberation of PAB by enzymes is given in Table II Autolysis of fresh Liver A on treatment with trypsin actually lowered the PAB potency from that of a water extract Other enzymes yielded some increases, a maximum of 50 per cent

TABLE II
Liberation of PAB from Liver A by Enzymes

Treatment	PAB
	γ per gm
Water extract, 1 hr, 15 lbs	1.6
Autolyzed, 37°, 3 days, toluene + CHCl ₃	0.8
Trypsin, 37°, 6 days, fresh enzyme daily	0.9
Pepsin, 50°, 3 hrs, pH 3.0	1.8
Papain, 37°, 24 " " 4.5	2.2
Taka-diastase, 37°, 24 hrs, pH 4.5	2.4

Fig. 2 contains typical curves for the hydrolysis of liver by acid and alkali at 15 pounds pressure All hydrolysates mentioned in this paper were brought approximately to pH 6.7 and filtered before assay Liberation of PAB by acid is rapid, however, we have never been able to obtain a PAB value of more than 4.4 γ per gm by acid treatment 2 N HCl for 1 hour gave optimum values HCl has generally given higher values than H₂SO₄ When acid hydrolysis was continued for longer than 1 hour, destruction of the vitamin became apparent Recovery of PAB added before hydrolysis with 2 N HCl was 70 per cent at 1 hour and 50 per cent at 6 hours

Alkaline hydrolysis at 15 pounds pressure is slower, but gives markedly higher final values The curve for 2 N NaOH had not reached a plateau even at 20 hours 5 N NaOH gave a maximum PAB content of about 9.0 to 9.5 γ per gm after 8 to 12 hours of hydrolysis Some destruction was noticeable on longer treatment Recoveries of PAB added to Liver A before alkaline hydrolysis were good 90 per cent recovery was obtained after 20 hours autoclaving with 2 N NaOH, and 109 per cent and 95 per cent after 4 and 8 hours respectively with 5 N NaOH

To obtain maximum values in more reasonable periods of time, hydrolysis

at 75 to 80 pounds pressure was tried Fig 3 shows these results With the higher pressure the maximum PAB liberation occurred in 1 hour with 5 N NaOH In separate experiments under these conditions PAB added

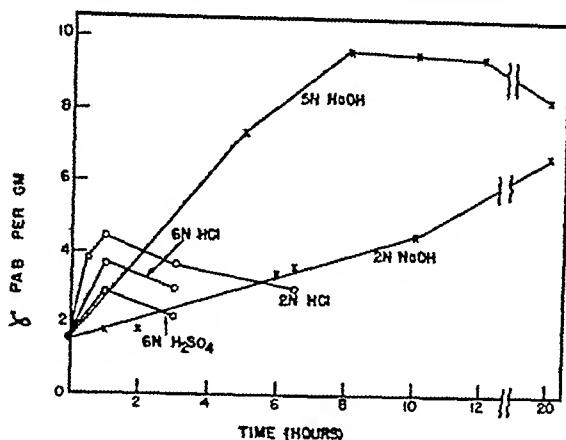


FIG 2 Liberation of *p*-aminobenzoic acid from Liver A

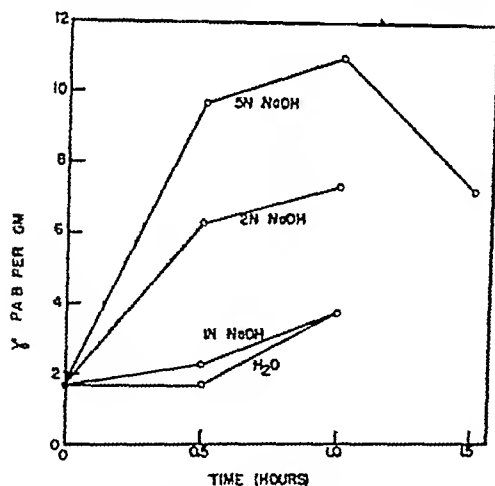


FIG 3 Hydrolysis of Liver A at 75 to 80 pounds pressure 50 mg samples in 3 cc of water or alkali were used

before hydrolysis was recovered 92 and 100 per cent Hydrolysis at high pressure with 0.1 N acid was ineffective and destruction occurred 0.1 N HCl for 1 hour gave a PAB value of 0.85 γ per gm, while 0.1 N H₂SO₄ yielded 1.25 γ per gm

The PAB of some water-soluble materials is also largely unavailable to the test organism (Table III). A water solution of the reference Difco yeast extract, while containing a large amount of bound PAB, did not change noticeably in potency on standing for 2 months at refrigerator temperature, hence hydrolysis does not appear to have occurred under these conditions. Acid or alkaline hydrolysis of a water extract of Liver A caused large increases in PAB values. A maximum of 6.7 γ per gm was reached. Also the assay of an HCl hydrolysate (2 N, 1 hour, 15 pounds) increased to 8.8 γ per gm after treatment with alkali, indicating that the acid treatment had extracted practically all of the PAB from the liver but had not rendered it available.

TABLE III
Liberation of PAB from Water-Soluble Materials

Material	PAB γ per gm			
	Original	2 N HCl 15 lbs, 1 hr	2 N NaOH 15 lbs 6 hrs	5 N NaOH 15 lbs
Difco yeast extract	80	103		120 (1 hr)*
Water extract of Liver A (per gm original material)	1.6	2.3	4.0	6.7 (1 ")*
HCl extract of Liver A (per gm original material)	4.4			8.8 (1 ")*
Urine	0.004†		0.325†	
"PAB-free" casein hydrolysate (per gm original material)	0			0.24 (1 hr) 2.6 (5 hrs) 1.0 (10 ")

* 75 to 80 pounds pressure

† Per cc

The casein hydrolysate listed in Table III was the "PAB-free" material used in the basal medium. Subsequent alkaline hydrolysis released 2.6 γ of PAB per gm of casein with destruction evident at 10 hours. Hydrolysis of urine with alkali also increased the PAB values. The acetyl derivative occurs here in considerable amounts. PAB recoveries of 105 to 120 per cent were obtained with the original and 85 to 110 per cent with the hydrolyzed material. These results suggest the suitability of this method for the determination of free and bound PAB in urine. Samples of N-acetyl PAB and of PAB ethyl ester were autoclaved with 2 N NaOH at 15 pounds pressure. Hydrolysis of both compounds was complete after 1 hour. Neither of these PAB derivatives has the properties of the difficultly hydrolyzed form of PAB.

Fig. 4 indicates the rate of liberation of PAB from Difco Bacto-peptone

by alkali. Here optimum values were obtained with 5 N NaOH by autoclaving for 1 hour at 75 to 80 pounds pressure.

The possibility exists that the additional PAB produced under the drastic alkaline conditions is an artifact due to the conversion of some "precursor" to PAB. The aromatic amino acids are obvious possibilities. In an effort to test this, 5 mg of tyrosine or of tryptophane were added to 50 mg samples of Liver A before hydrolysis with 5 N NaOH at 75 pounds for 1 hour. The control tube with Liver A alone assayed 8.8 γ of PAB per

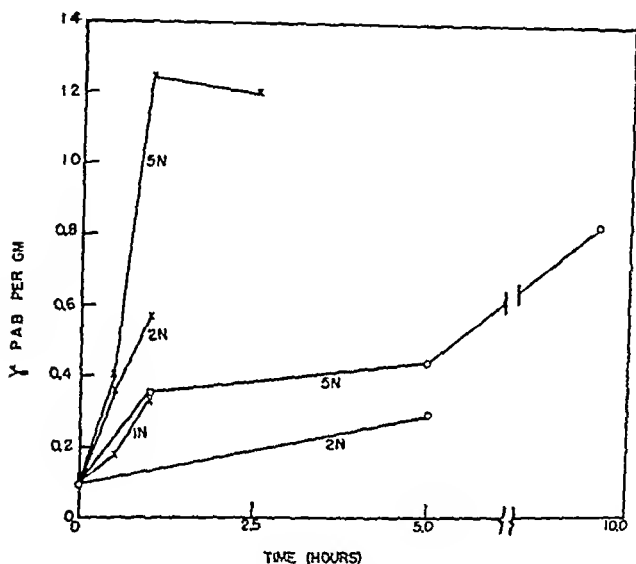


FIG 4 Liberation of *p*-aminobenzoic acid from peptone by NaOH. 50 mg samples in 3 cc of alkali were used. O, 15 pounds pressure, X, 75 to 80 pounds pressure.

gm after hydrolysis, while with added tyrosine or tryptophane values of 9.2 and 7.5 γ per gm respectively were obtained. A mixture of eighteen amino acids simulating hydrolyzed casein was prepared. This assayed 0.12 γ of PAB per gm before treatment, and 0.27 γ per gm after 1 hour in 5 N NaOH at 75 pounds pressure. When 12 mg of the amino acid mixture were added to 50 mg of Liver A before a similar hydrolysis, 9.5 γ of PAB per gm were found. A control tube with Liver A alone yielded 10.0 γ per gm. These results indicate that the common amino acids are not acting as PAB "precursors."

The phenomenon of rapid but incomplete liberation of PAB by acid and slower but more complete action by alkali indicates the possibility that the liberation of at least a portion of the PAB is a two-step process. By

this concept, acid would hydrolyze rapidly certain linkages which alkali could attack only slowly. However, once these bonds had been opened the remaining linkages would be split more completely by alkali than by acid.

To test this, an acid hydrolysate of Liver A was again hydrolyzed with alkali. The material had been autoclaved 40 minutes with 2 N HCl at 15 pounds pressure. It assayed 4.1 γ of PAB per gm. 80 minutes hydrolysis at 15 pounds pressure with 1 N, 2 N, and 5 N NaOH raised this value to 5.1, 5.7, and 8.1 γ of PAB per gm. respectively. Comparable treatment of Liver A with alkali yields less than 3 γ per gm. The figures indicate that alkaline hydrolysis is more rapid after a preliminary acid treatment.

Destruction of PAB—PAB destruction during acid hydrolysis of liver and of casein hydrolysate has already been mentioned. Slow destruction also occurs on long alkaline hydrolysis (Figs 2 and 3). PAB is generally more stable to alkali than to acid. In experiments with the pure material, 40 to 50 per cent destruction occurred in 4 N HCl on autoclaving for 1 hour at 15 pounds pressure, and 0 to 15 per cent in 2 N NaOH during 6 hours at 15 pounds. At 75 to 80 pounds pressure over 99 per cent destruction resulted from use of 0.1 N HCl for 1 hour. As previously mentioned, no loss of added PAB during hydrolysis with 5 N NaOH in the presence of liver could be demonstrated after 1 hour at 75 to 80 pounds pressure.

DISCUSSION

It is apparent that PAB occurs in many natural materials in an extremely resistant combination. Part of the PAB is in a compound resistant to acid hydrolysis. This is demonstrated by our inability ever to free more than 4.4 γ of PAB per gm. of Liver A by acid treatment, while alkali gives values up to 11 γ per gm. Also the "PAB-free" acid hydrolysate of casein (hydrolyzed 24 hours with 20 per cent HCl) still contained PAB, as determined by alkaline hydrolysis.

While the possibility is not ruled out that the PAB obtained after alkaline hydrolysis is an artifact, our inability to demonstrate any increase in PAB after hydrolysis of the amino acid mixture either alone or added to Liver A argues against it. The excellent recoveries of PAB obtained with alkaline hydrolysis preclude explanation of this increase as conversion of PAB to a more active form (as in the transformation of pyridoxine to pseudopyridoxine (15)).

For most materials we would recommend treatment with 5 N NaOH, preferably at 75 to 80 pounds pressure for 1 hour. However, it would be wise to investigate less drastic conditions for any new material, especially if the acetyl compound is the main bound form of PAB present, as in blood or urine.

The values obtained by our procedures may still not be the total PAB,

but the conditions outlined give maximum values. The availability of these various forms of PAB to the animal is still unknown.

SUMMARY

1. A microbiological assay for PAB in which *Clostridium acetobutylicum* S9 is employed as the test organism is described. The assay range is 0.3 to 1.5 millimicrograms of PAB per 10 cc. Growth is determined by turbidity at 20 to 24 hours. The accuracy is ± 10 per cent.

2. The PAB in liver occurs in a bound form from which it may be freed by alkali, but only partially by acid. Enzyme treatment was not successful. Autoclaving at 75 to 80 pounds for 1 hour in 5 N NaOH gives maximum PAB content. Water-soluble materials as well contain PAB in an unavailable form from which it is again best released by alkaline hydrolysis.

3. PAB is rapidly destroyed during acid hydrolysis. It is relatively stable to alkali.

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STUDIES IN THE METABOLISM OF PURINES AND PYRIMIDINES BY MEANS OF ISOTOPIC NITROGEN*

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During an investigation of nitrogen metabolism in birds (1) it was found that, when isotopic ammonia was administered, N^{15} was incorporated in the nucleic acids. The values obtained suggest that nucleic acids are synthesized even more rapidly than proteins. Since uric acid is a major end-product in avian metabolism, the rapid rate of nucleic acid turnover suggested that it may play an intermediate role in the general nitrogen metabolism. The present investigation deals with the interrelationship of purines, pyrimidines, and creatine and their possible function as precursors for nucleoproteins.

If dietary purines can serve in the synthesis of nucleoproteins, administration of isotopic purines must result in their deposition in the nucleic acids as an intermediary step and, finally, in their appearance as nitrogenous end-products. Since the nucleic acids also contain pyrimidines, a similar reasoning applies to the metabolism of these substances. The classical feeding experiments of Cerecedo and coworkers (2-6) indicate that, with the exception of cytosine, which is poorly absorbed, these compounds are oxidized and excreted as urea and oxalic acid.

The nitrogen of isotopic guanine, when administered to rats, is excreted as allantoin and, to an almost insignificant extent, as urea and ammonia (Table I). Practically no isotope could be found in the purines or pyrimidines isolated from nucleic acids. Essentially the same result was obtained with pigeons that had received isotopic guanine, the purine analogue in this case being uric acid. Tissue creatine, isolated as creatinine, contained little or no isotope marker, but the nitrogenous end-product, uric acid, was high. Neither the purines nor the pyrimidines isolated from nucleic acids showed the expected isotope concentration, dietary purines, therefore, do not appear to be incorporated as such into the tissues. When isotopic creatine was fed to rats over a period of 6 days, the allantoin

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isolated from the urine contained no N^{15} . Similarly, uric acid isolated from the droppings of pigeons that had received isotopic creatine (sarcosine as well as amidine N^{15}) was found to be free of isotope marker. The results of this experiment are given in Table II and show that the only appreciable

TABLE I

N^{15} Content of Substances Isolated after Administration of Isotopic Guanine

The amounts of isotopic guanine administered are recorded in the experimental part under "Guanine feeding," Experiments I, II, and III. The isotope concentration was computed on the basis of 100 per cent in the test substance. On this basis the average error of a determination is ± 0.07 atom per cent.

	Experiment I rats	Experiment II rats	Experiment III pigeons
Ammonia	0.74		
Allantoin	13.9		
Urea	0.54		
Combined purines	0.05	0.10	0.20
Protein hydrolysate	0.15		
Guanine	0.02	0.20	
Adenine	0.25	0.12	
Cytosine	0.32		
Arginine	0.22		
Glutamic acid	0.27		
Tissue creatine	0.30		0.25
Uric acid			1.30

TABLE II

N^{15} Content of Substances Isolated after Administration of Isotopic Creatine and Guanidoacetic Acid to Pigeons

The values were computed on the basis of 100 atom per cent N^{15} excess in the test substance fed.

	Creatine amidine N^{15}	Creatine sarcosine N^{15}	Guanidoacetic acid
Combined purines	0.33	0.03	0.12
Tissue creatinine	3.95	2.40	2.64
Uric acid	0.57	0.12	0.36
Nucleic acids	0.21	0.15	0.03
Average error	0.10	0.10	0.10

isotope concentration was found in the tissue creatine. The replacement appears to proceed at approximately the same rate as in mammals (7). Similar results were obtained with isotopic guanidoacetic acid. When this was fed to pigeons, only a negligible amount of N^{15} appeared in the uric acid isolated from the droppings, but the tissue creatine contained the expected amount.

Upon administration of isotopic uracil and thymine to rats, the only appreciable isotope concentration was found in urinary ammonia and urea. The most significant result of these experiments (Table III) is the low value for allantoin compared to that of urea, which rules out biological conversion of pyrimidines to purines.

Synthesis of Isotopic Guanine—Guanine was synthesized according to the procedure of Traube (8) in which, as in his improved synthesis of 2,4-diamino-6-hydroxypyrimidine (9), guanidine was used as starting material. This was regarded as a convenient way of introducing isotopic nitrogen by way of the well established synthesis (7) of cyanamide from isotopic ammonia and normal cyanogen bromide. When cyanamide and

TABLE III

N¹⁵ Content of Substances Isolated after Administration of Isotopic Uracil and Thymine to Rats

The values are computed on the basis of 100 atom per cent N¹⁵ excess in the test substance fed

	Uracil	Thymine
Combined purines	0 27	
Ammonia	0 71	1 0
Allantoin	0 01	0 09
Urea	1 21	1 49
Guanine	0 10	
Adenine	0 11	0 20
Cytosine	0 32	0 23
Thymine	0 23	0 30
Creatinine		0 06
Protein hydrolysate	0 23	0 22
Feces	0 29	0 41

ammonium chloride (10) were heated together in ethyl alcoholic solution in bomb tubes at 100° for periods of 5 to 7 hours, no reaction occurred, and, with longer heating or higher temperatures, the cyanamide polymerized to dicyanodiamide. On the other hand, guanidine was readily produced by heating ammonium bromide and cyanamide in the presence of 1 mole of excess ammonia in a bomb tube for 96 hours at 100°. A reasonably pure product and a 96 per cent recovery of the excess isotopic ammonia were secured. Little or no polymerization took place, probably because the excess ammonia raises the pH of the solution sufficiently above that at which polymerization of cyanamide takes place most rapidly (11, 12), namely, 9.7. The addition of small amounts of bromine or iodine was found to catalyze the reaction. This addition was later abandoned, as the bromo- or iodoguanidine formed as by-products were difficult to remove.

The guanine finally prepared contained 4.03 atom per cent N^{15} excess with the isotope in the 2-amino group and the 2 pyrimidine nitrogen atoms.

In order to establish the biological origin of substances containing ring systems with more than 1 marked nitrogen atom it becomes necessary to degrade the material and to isolate its split-products. In contrast to similar examples reported previously the amount of starting material in our instance was extremely small. The amount of pure material isolated from nucleic acids (adenine and guanine) ranged from 50 to 150 mg., depending on the number of animals used. Extensive study of such degradations seemed necessary at the time. Strecker oxidized guanine with chloric acid (13) to guanidine and parabanic acid. We were unable to perfect the technique of this reaction to the point where parabanic acid could be isolated from the oxidation mixture of 50 mg. of guanine or less, but guanidine was isolated as the picrate. When this reaction was carried out with guanine synthesized as described above, the guanidine was found to contain N^{15} in the exact amount demanded by the theory.

Another degradation was the deamination of guanine to xanthine. According to the procedure of Fischer (14) xanthine was obtained in good yield from 50 mg. of guanine as starting material, even after purification through its silver salt. It contained the calculated amount of isotope marker. These two experiments together with the synthesis of guanine give sufficient proof for the correctness of the position of N^{15} in the molecule. At the same time, these experiments show that splitting of the purine ring system with chloric acid occurs between the 1-6 and 3-4 atoms, it also gives added evidence for the existence of cyanamide in its mesomeric form (7, 15).

Synthesis of Isotopic Thiourea—The reaction of cyanamide and hydrogen sulfide (16) in acid medium with Sb_2S_3 as catalyst was used. The final product was recrystallized from *n*-propyl alcohol until its correct melting point (174°) was obtained.

Synthesis of Isotopic Thymine—Thymine was prepared from thiourea and ethyl formylpropionate according to the procedure of Wheeler and Liddle (17). Isotopic thiourea was condensed with the sodium salt of ethyl formylpropionate in methanolic sodium methylate solution to 2-mercapto-5-methyl-6-hydroxypyrimidine which, when treated with concentrated hydrochloric acid, gave thymine in almost quantitative yield.

Synthesis of Isotopic Uracil—Although the simplest method for the preparation of uracil seems to be the condensation of urea with malic acid (18), we have synthesized uracil from thiourea and ethyl formylacetate according to Wheeler and Liddle (17), because the resulting compound, 2-mercapto-6-hydroxypyrimidine, could also be used as an intermediate in the synthesis of cytosine. Circumstances, however, prevented us from

preparing cytosine 2-Mercapto-6-hydroxypyrimidine on treatment with concentrated hydrochloric acid gave uracil, 2,6-dihydroxypyrimidine, in 94 per cent yield

EXPERIMENTAL

Guanidine Hydrobromide (I)—4.77 gm (3 moles) of isotopic ammonium chloride, 10.1 atom per cent N^{15} excess, were decomposed according to the procedure previously reported, special care being taken to exclude moisture. The ammonia was carried into a dry ice-cooled bomb tube containing 20 cc of absolute ethyl alcohol. A gas inlet tube, especially constructed for this purpose, so as to fit the bomb tube, was used. The small amount of ammonia that escaped was caught in a trap containing dilute sulfuric acid. When the absorption of ammonia in the alcohol was complete, the bomb tube was allowed to warm up to 0° and kept at this temperature in ice. A solution of 3.18 gm of freshly prepared cyanogen bromide in 5 cc of absolute ethyl alcohol was added, and the tube sealed and heated to $105\text{--}110^{\circ}$ for 96 hours.

The length of time necessary for this reaction was determined by running a series of experiments with non-isotopic ammonia. The tubes were heated to 110° for various lengths of time and the contents tested with ammoniacal silver nitrate for the presence of cyanamide. At the end of 96 hours the test for cyanamide was consistently negative.

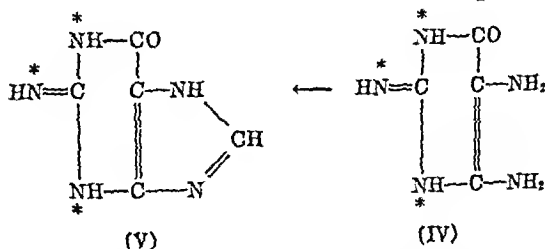
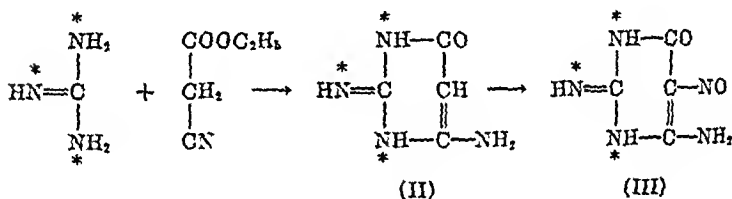
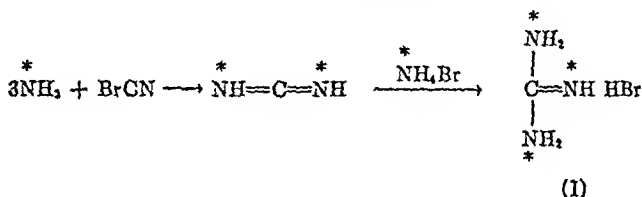
The tube was cooled in ice, opened, cut off as straight as possible, and the open end fire-polished. The gas inlet tube was inserted, and the excess ammonia blown off by a slow stream of nitrogen and collected in an acid trap. 94 to 96 per cent of the excess ammonia (10.1 atom per cent N^{15} excess) could thus be collected. The clear alcoholic solution was slowly evaporated to dryness and the residue dried *in vacuo* at room temperature. 4.02 gm of guanidine hydrobromide were collected. Although this product was not analytically pure, it was found to be quite satisfactory for the following condensation.

2,4-Diamino-6-hydroxypyrimidine (II)—This compound was prepared according to Traube (9) with methyl alcohol instead of ethyl alcohol and 50 per cent excess cyanoacetic ester, 5.21 gm of guanidine hydrobromide yielded 4.08 gm of diaminohydroxypyrimidine sulfate.

Alloxan-2,4-diamide-5-oxime (III)—This compound was prepared in 99.8 per cent yield as described by Traube (8), 3.45 gm of the isonitroso compound were obtained from 4.08 gm of diaminohydroxypyrimidine sulfate.

2,4,5-Triamino-6-hydroxypyrimidine (IV)—The original procedure of Traube was slightly modified to yield the sulfate directly instead of the free base. 3.45 gm of the isonitroso compound were finely powdered in a large

agate mortar and slowly added to 50 cc of boiling water containing 15 cc of commercial ammonium sulfide. This suspension was kept boiling for 3 hours with occasional stirring and addition of 3 to 5 cc of ammonium sulfide and the necessary amount of water to keep the volume constant. When all the nitroso compound was reduced, the solution was immediately filtered. On prolonged boiling in the absence of excess sulfide the solution turned pink. This could not be prevented by carrying out the reaction in an atmosphere of nitrogen. The solution was filtered by suction directly into 75 cc of 3 N H₂SO₄ cooled in ice. 4.68 gm (82 per cent) of triamino hydroxypyrimidine sulfate were obtained in the form of light yellow crystals. No further purification was necessary.

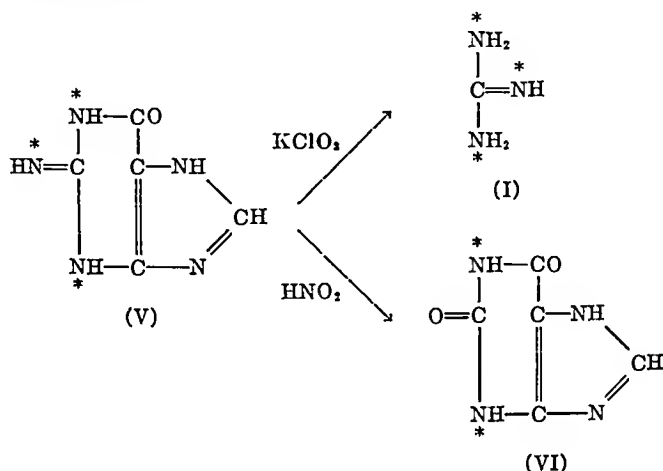


Guanine Sulfate (V)—4.62 gm of triamino hydroxypyrimidine sulfate and 4.0 gm of anhydrous sodium formate were refluxed with 50 cc of 90 per cent formic acid for 8 hours. The solution was filtered and evaporated on the steam bath. The residue was dissolved in dilute sulfuric acid, boiled with charcoal for 5 hours, and the product crystallized. The mother liquors in each case were neutralized to pH 5, the precipitated free base

removed by filtration and redissolved in a smaller volume of dilute sulfuric acid, and the guanine again crystallized as sulfate. On repetition of this procedure, a total of 2.94 gm of guanine sulfate was obtained.

Analysis— $(C_5H_5N_5O)_2H_2SO_4 \cdot 2H_2O$ Calculated, N 32.8, found, N 32.8

The compound contained 4.03 atom per cent N^{15} excess, calculated 4.04 atom per cent N^{15} excess

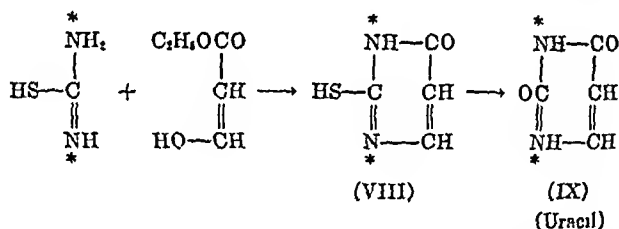
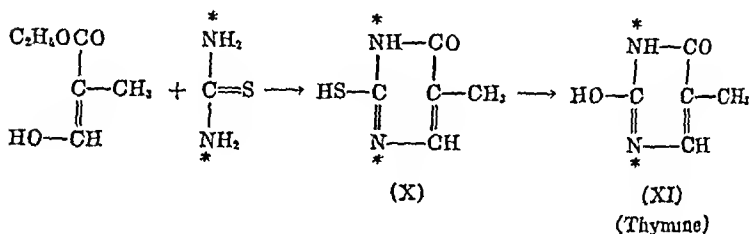
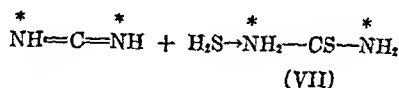


Degradation of Isotopic Guanine to Guandine—50 mg of free guanine (4.03 atom per cent N^{15} excess) were prepared from the sulfate. The free base was suspended in 40 cc of HCl and 32 mg of $KClO_3$ added in small amounts over a period of 1 week. At the end of this time, the solution was colorless and all solid material had gone into solution. It was evaporated to dryness under reduced pressure and extracted five times with absolute alcohol. The clear alcoholic solution was concentrated to 10 cc. A small amount of semicrystalline material, probably parabanic acid, was removed by filtration. The filtrate was evaporated to dryness, the residue dissolved in 20 cc of water, and 20 cc of saturated picric acid added. After the material had stood in the ice box overnight, 32 mg of a crystalline picrate precipitated which, when recrystallized from hot water, gave analytical values corresponding to those of guandine picrate.

Analysis— $C_7H_5O_7N_6$ Calculated, N 29.2, found, N 29.4

20 mg of this picrate were decomposed with dilute hydrochloric acid and the picric acid continuously extracted with ether. Isotope analysis indicated 6.92 atom per cent N^{15} excess, calculated 6.76 atom per cent N^{15} excess.

Degradation of Isotopic Guanine (V) to Xanthine (VI)—32 mg of guanine (4.03 atom per cent N^{15} excess) were dissolved in 2.0 cc of 4 N sulfuric acid and heated in a beaker of boiling water, 24 mg of sodium nitrite were added in very small amounts, and the solution neutralized and cooled in ice. The dark colored xanthine which precipitated was purified by transformation into its silver salt which was decomposed with dilute hydrochloric acid. On neutralization to pH 5 xanthine crystallized in analytically pure form. It contained 3.44 atom per cent N^{15} excess, calculated 3.38 atom per cent N^{15} excess.



Isotopic Thiourea (VII)—2.67 gm of isotopic cyanamide prepared according to the procedure previously reported (7) were dissolved in 70 cc of water, and 5.34 gm of antimony pentasulfide and 0.7 cc of concentrated hydrochloric acid added. The mixture was heated to 90° on the steam bath and a rapid stream of hydrogen sulfide blown through the suspension for 2 hours. After removal of the excess hydrogen sulfide by boiling, the antimony pentasulfide was filtered off and washed with hot water. The filtrate was treated with 20 mg of potassium carbonate and cleared by filtration through a small amount of infusorial earth. It was then evaporated to dryness under reduced pressure and the residue recrystallized from *n*-propyl alcohol until its melting point was 174°. The total yield after concentration of mother liquors was 2.14 gm. The material contained 5.28 (calculated 5.25) atom per cent N^{15} excess.

Isotopic Thioracil (VIII)—2.14 gm of isotopic thiourea were added to

100 cc of 95 per cent ethyl alcohol in which 1.5 gm of sodium had been dissolved. To this mixture 12 gm of powdered ethyl sodium formyl acetate were added and the solution refluxed for $3\frac{1}{2}$ hours. At the end of this time, 20 cc of water were added and refluxing was continued for another half hour. The mixture was then brought to dryness under reduced pressure and redissolved in 40 cc of water, chilled in ice, and acidified with 50 per cent acetic acid. After being left to stand in the ice box overnight, the product was filtered off and recrystallized from 125 cc of boiling water, charcoal being used to remove colored by-products. 1.57 gm of thiouracil were obtained.

Isotopic Uracil (IX)—The procedure employed was exactly the same as that described by Wheeler and Liddle (17). Analytically pure uracil was obtained in 96 per cent yield. The final product contained 5.42 atom per cent N^{15} excess, the calculated amount was 5.25 atom per cent N^{15} excess.

Isotopic Thiothymine (X)—2.0 gm of isotopic thiourea (5.28 atom per cent N^{15} excess) were condensed with 12 gm of sodium ethyl formylpropionate in the manner described for thiouracil. After two recrystallizations from boiling water 940 mg of thiothymine were obtained.

Isotopic Thymine (XI)—940 mg of thiothymine were treated with hydrochloric acid as described by Wheeler and Liddle (17). 760 mg of thymine were obtained. The product contained 4.88 atom per cent N^{15} excess, the calculated amount was 5.25 atom per cent N^{15} excess.¹

Feeding Experiments

The experimental conditions employed in the feeding experiments were identical with those previously reported (7). Rats received the same stock diet. Pigeons received an adequate diet of mixed grains, the isotopic compounds were dissolved in water and administered by stomach tube every 6 hours. Guanine was administered as the hydrochloride, since the free base is insoluble in water. Urinary ammonia, urea, muscle creatine, and amino acids were isolated by the usual procedures. Allantoin was isolated according to Wiechowski (19). The isolation of purines and pyrimidines will be described in the following representative experiment.

Nucleic Acids (General Procedure)—Combined internal organs (liver, kidney, spleen, pancreas, testes, thymus gland) were immediately minced

¹ Although somewhat exceeding the experimental error, the discrepancy between these values can be explained as follows. After feeding experiments and elementary analyses had been carried out, an extremely small amount of material was available for spectrographic analysis, necessitating a dilution with a known amount of normal nitrogenous material. The error involved in this particular incidence may well account for the difference of 5.25 and 4.88 atom per cent N^{15} excess.

and suspended in 95 per cent ethyl alcohol overnight. The precipitate was filtered and extracted with absolute alcohol in a Soxhlet apparatus for 6 hours. The alcohol was then replaced with ether and extraction continued for another 6 hours. Adhering solvent was removed in a stream of dry air and the fat-free product powdered in a mortar. 100 gm of wet organs usually gave about 33 to 35 gm of dry material. A small sample of this powder was used for the determination of "combined purines" according to Graff and Maculla (20) and the rest extracted with 10 per cent sodium chloride. The mixture was occasionally heated on the steam bath with stirring over a period of 2 to 3 days and finally filtered in a steam-heated Buchner funnel. This extraction was repeated three times and the sodium salts of the nucleic acids precipitated by addition of 2.5 volumes of ethyl alcohol. The salts were centrifuged off and washed several times with absolute alcohol and ether. In this way, 2.4 to 3.0 gm were obtained from 35 gm of dry tissue. The sodium salts were dissolved in 100 cc of water by warming the mixture on the steam bath and stirring at high speed.

The solution was then placed in an ice bath (stirring continued) and 5.0 cc of concentrated hydrochloric acid added drop by drop. The precipitated nucleic acids were centrifuged, washed with water, alcohol, ether, and finally with absolute ether. The product was almost pure white and practically free of protein, its weight was approximately one-third that of the original sodium salt.

Nucleic Acid Splitting—The following representative procedure is a modification of the usual hydrolysis of nucleic acids (21). The procedure was successfully used for the isolation of guanine, adenine, cytosine, and thymine with as little as 450 mg of nucleic acids. In the original study of the method nucleic acids prepared from beef pancreas were used, which were found to differ appreciably from rat nucleic acids in their relative adenine and guanine content. The latter was found to contain less than one-half the amount of adenine, with a corresponding increase in guanine (Table IV).

1.0 gm of the finely powdered and carefully dried nucleic acids was suspended in 12 cc of absolute methyl alcohol and dry hydrochloric acid blown through the solution for exactly 3 hours. The solution turned wine-red, the precipitate dissolved, and finally the purines precipitated as hydrochlorides. The flask was stoppered (glass) and allowed to stand in the ice box for 24 hours. The ice-cold solution was again saturated with dry HCl, and the precipitate centrifuged and washed with methyl alcohol saturated with HCl until the washings were colorless. Combined washings and supernatant were set aside for the isolation of pyrimidines. After being dried at 100°, 300 mg of purine hydrochlorides were obtained. The purine hydrochlorides were dissolved in 5.0 cc of water and heated

with charcoal, filtered, and neutralized with dilute NaOH, when acid to litmus and alkaline to Congo red, the guanine precipitated as an amorphous powder. After having remained in the ice box for 3 days, it was centrifuged and the supernatant solution set aside for the isolation of adenine. The free guanine was dissolved in dilute sulfuric acid and crystallized as sulfate in the manner described in the synthesis of isotopic guanine. Approximately 160 mg of guanine sulfate were obtained.

The supernatant liquid from the precipitation of guanine was combined with the washings and concentrated to about 30 cc. A small amount of insoluble material was removed by filtration and an equal volume of saturated picric acid added. A copious precipitate of adenine picrate formed, which was recrystallized from a minimum amount of 25 per cent acetic acid until the correct analytical figures were obtained (29.2 per cent N). Usually 60 to 70 mg of adenine picrate were obtained after three recrystallizations.

TABLE IV
Comparison of Rat and Beef Nucleic Acids

The values given are of mg of substance isolated according to the procedure outlined in the text.

	10 gm. beef nucleic acids	10 gm. rat nucleic acids
Guanine sulfate	88	164
Adenine picrate	180	61
Thymine	36	12
Cytosine picrate	36	10
Total purines as hydrochlorides	301	291

zations, approximately 10 per cent was lost on each recrystallization. N^{15} analyses were carried out in the manner described above for guanine picrate.

The methanolic solution containing pyrimidine nucleosides was taken to dryness and the dark colored residue suspended in 15 cc of 20 per cent HCl, sealed in a bomb tube, and heated at 185° for 3 hours. The contents were filtered and the filtrate evaporated to dryness. The residue was digested with 20 cc of water, filtered, and acidified with 1 drop of concentrated HNO_3 . Undesirable by-products, purines, phosphates, etc., were removed by addition of 50 cc of 10 per cent silver nitrate, the precipitated silver salts carefully washed, and the combined filtrate and washings concentrated to less than 30 cc. The bulk of thymine crystallized out in the form of long needles. After repeated recrystallization from water, the yield was 10 to 15 mg.

The filtrate from thymine was neutralized with barium hydroxide and

any precipitate which formed below pH 7 was discarded. On further addition of barium hydroxide pyrimidine silver precipitated together with some Ag_2O . The precipitated silver salts were carefully washed until free from alkali, suspended in 20 cc of water, and decomposed with hydrogen sulfide. The clear filtrate from Ag_2S was concentrated to 10 cc and 20 cc of saturated picric acid were added. Cytosine picrate precipitated immediately and was recrystallized from hot water until analytically pure. For N^{15} analyses the picrate was decomposed as described above.

Guanine Feeding Experiment I—Four adult male rats with a combined weight of 1273 gm were fed 158 mg of guanine hydrochloride (4.03 atom per cent N^{15} excess) over a period of 3 days. The amount of guanine was calculated to be approximately equivalent to the amount of allantoin excreted. The results of this experiment are recorded in Table I.

Experiment II—This experiment was identical with Experiment I except that the animals received twice the amount of guanine hydrochloride per kilo per day. The results are recorded in Table I.

Experiment III—Two adult pigeons with a combined weight of 810 gm were fed 54 mg of guanine hydrochloride per kilo per day. The animals were killed 6 hours after the last feeding, and purines, creatine, nucleic acids, and uric acid were isolated. The analytical values are recorded in Table I.

Creatine Feeding Experiment I—Four adult male rats weighing 1215 gm were fed 44 mg of creatine hydrate per kilo per day for 6 days. The urines from the first 4 days were pooled and allantoin isolated. The samples of allantoin from the last 2 days of Animals 1 and 2 as well as 3 and 4 were found to have identical isotope content, namely, 0.004 atom per cent N^{15} excess. No isotope was found in the combined purines isolated from the internal organs. The creatinine hydrate was prepared as previously reported (15) and contained 3.31 atom per cent N^{15} excess in the amidine group only.

Experiment II—Four adult pigeons with a combined weight of 1402 gm were fed 60 mg of creatine hydrate per kilo per day for 3 days. The creatine hydrate contained 3.31 atom per cent N^{15} excess, with the isotope marker in the amidine group only. Of the substances isolated only the muscle creatine contained isotope, and a degradation with baryta showed that all of it was located in the amidine group, the sarcosine moiety containing none. A protein hydrolysate and arginine isolated from it did not contain any isotope. The analytical values are recorded in Table II.

Experiment III—Two adult pigeons with a combined weight of 872 gm were fed 44 mg of creatine hydrate per kilo per day for 3 days. The creatine was prepared from isotopic sarcosine and normal cyanamide and contained 3.34 atom per cent N^{15} excess. The combined purines, creatine, and uric acid were isolated. The results are tabulated in Table II.

Guanidoacetic Acid Feeding—Two adult pigeons with a combined weight of 710 gm were fed 47 mg of guanidoacetic acid (3.34 atom per cent N^{15} excess) per kilo per day for 3 days. The combined purines, uric acid, creatinine, and nucleic acids were isolated. The results are recorded in Table II.

Uracil Feeding—Eight adult male rats with a combined weight of 265 gm were fed 30 mg of isotopic uracil (5.48 atom per cent N^{15} excess) per rat per day for 3 days. The results of this experiment are given in Table III.

Thymine Feeding—Four adult male rats with a combined weight of 1200 gm were fed 33 mg of isotopic thymine (4.88 atom per cent N^{15} excess) per rat per day for 3 days. The results are given in Table III.

DISCUSSION

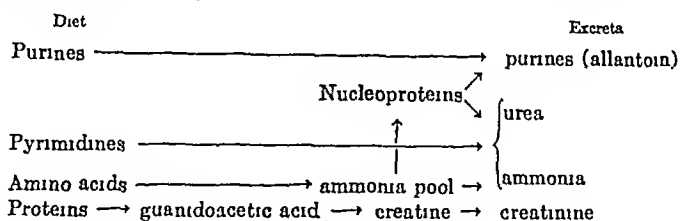
In investigations carried out in this laboratory over a period of 6 years, it has been found that the addition to the diet of substances which are normal tissue constituents leads to their deposition as an inseparable mixture of the preformed and dietary material. In contrast to this, the nitrogenous bases of nucleic acids, the purines and pyrimidines, do not enter the structure of the nucleus but are immediately metabolized to their respective end-products. This exception to the rule is difficult to explain. Since these bases are metabolized in a normal fashion, it must be assumed that they enter the cell specific for this transformation and that the metabolic changes take place in the cytoplasm. This may be due either to a certain specificity of the nuclear membrane or the inability of the nucleus to utilize preformed dietary purines and pyrimidines for the synthesis of nucleic acids and nucleoproteins. In view of the fact that there is considerable evidence against the concept of two independent types of catabolisms, the specificity of the nuclear membrane is the more plausible explanation.

The finding (Table II) that creatine and guanidoacetic acid are ineffective as purine precursors and are not metabolized to uric acid illustrates the biological inertia of creatine and its precursors in birds. In the pigeon, as in the rat, the conversion of guanidoacetic acid into creatine appears to be unidirectional.

In contrast, the results of the pyrimidine experiments do not lead to so clear a conclusion. The pyrimidines themselves, although almost quantitatively absorbed when administered in small amounts, are not deposited in the nucleus but are immediately metabolized to urea and ammonia. It is entirely conceivable that the truly nuclear pyrimidines are catabolized in the same way but it is quite possible that they represent intermediates in the biological synthesis of purines in the nucleus if the starting materials are relatively simple substances, such as ammonia and small organic molecules.

The biological changes of purines and pyrimidines here reported represent a "cytoplasmic" catabolism and throw no light upon the "nuclear" synthesis of purines

The findings here reported, like those of Barnes and Schoenheimer (1) indicate that all amino acids except lysine (22) participate in the synthesis of purines, and that neither histidine nor arginine makes any outstanding contribution to it. The hypothetical conversion may be summarized by the accompanying diagram



SUMMARY

1 Isotopic guanine was prepared which contained isotopic nitrogen in nitrogen atoms 1 and 3 of the ring, as well as in the free amino group at position 2

2 No connection between the creatine cycle and purine and pyrimidine metabolism could be detected

3 The conversion of uracil and thymine to urea and ammonia, observed by Cerecedo and coworkers, was confirmed

4 Evidence was presented that pyrimidines are completely metabolized when administered in small amounts

5 Guanidoacetic acid was shown to be an effective creatine precursor in birds. It does not act as a purine or pyrimidine precursor

6 Neither purines nor pyrimidines supplied in the diet are utilized by the body for the synthesis of nucleoproteins. The belief is expressed that they are either synthesized within the nucleus from smaller molecules or they are utilized for such a synthesis if they are supplied as nucleosides, nucleotides, or nucleic acids

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CARBOHYDRATE METABOLISM IN VITAMIN B₁ DEFICIENCY*

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The fundamental observations of Peters (1) and his school disclosed that vitamin B₁ deficiency studied in pigeons is associated with inability to oxidize pyruvic acid. These observations stimulated interest in the relationship between pyruvic acid and carbohydrate metabolism. Among the important contributions are those of Lohmann and Schuster (2) and Banga, Ochoa, and Peters (3, 4) who showed that diphosphothiamine was the coenzyme for carboxylase. The inability to oxidize pyruvic acid in the absence of vitamin B₁ has been examined in many species including the human being. Platt and Lu (5) showed that the severity of beriberi in human subjects could be correlated with the concentration of pyruvic acid in the blood. When they used exercise as a means of increasing the concentration of pyruvic acid in the blood, they observed that its rate of removal was slackened in vitamin B₁-deficient subjects (6). Voit and coworkers (7) have found that in other conditions associated with acute vitamin B₁ deficiency, namely Wernicke's syndrome and acute peripheral neuropathy, pyruvic acid accumulates in the blood stream.

The normal level of pyruvic acid in the blood is low not only because the tissues oxidize it rapidly, but also because some of it is reduced to lactic acid. The observations of Stotz and Bessey (8), of Friedemann and Barboraka (9), and of Klein (10) indicate a more or less constant equilibrium between lactic acid and pyruvic acid. In humans the ratio is approximately 12:2 and in rats 9:1 (8). In the present observations the relationship of the concentrations of blood sugar, lactic acid, and pyruvic acid was determined under two conditions, first, after the injection of glucose, and second, after the injection of pyruvic acid. The tests were performed first after the dogs had been on a complete dietary. Later while these same animals were ingesting a diet devoid of vitamin B₁, they were tested twice, once before they showed signs of acute deficiency and again later when they were *in extremis*.

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Method

Dietary—Sixteen healthy adult dogs were carefully chosen. They were placed on the following diet similar to that of Street, Cowgill, and Zimmerman (11): Labco vitamin-free casein 23 per cent, sucrose 50, Crisco¹ 20, bone ash 4, Food Research Laboratories salt mixture (12) 3. Brewers' yeast when given was added to the diet in an amount equal to 8 per cent of the weight of the diet.

A portion of this diet equivalent to 90 calories per kilo was carefully weighed out daily for each dog, the amount recalculated weekly in accordance with change in weight.

This vitamin-free diet was supplemented with the following synthetic vitamin mixture, given by capsule three times weekly per kilo of body weight per day: thiamine 0.05 mg, riboflavin 0.10 mg, niacin 2.00 mg, pyridoxine 0.06 mg, calcium pantothenate 0.25 mg, choline 50.00 mg, inositol 0.30 mg, *p*-aminobenzoic acid 0.30 mg, vitamin A 857 units, vitamin D 107 units. 100 mg of vitamin C and 1 mg of vitamin K were given each dog orally every 2 weeks.²

During the control period of 5½ months while the sixteen dogs were on the above diet supplemented with yeast and vitamins as indicated, they all showed a moderate gain in weight and were in excellent condition. They were under close observation and no difference from dogs receiving the standard dog diet could be detected. After this period of alimentation, six of the animals were retained as controls and the other ten were used as test animals for acute vitamin B₁ deficiency. Two of the controls continued to receive the diet plus 8 per cent yeast and vitamins as previously. The other four control dogs were matched with four of the test animals. Their diet contained no yeast and differed from that of the test animals only in that their vitamin capsules contained 50 γ per kilo per day of thiamine, in addition to the other vitamins, whereas the vitamin capsule of the test animals contained no thiamine at all. Each day the diet of the control dogs contained the same number of calories as were ingested by the dog with which they were matched on the previous day.

Chemical Determinations—After the sixteen animals had been on the complete dietary for 3½ months, control values were established for them for the effects of the injection of glucose on the blood levels of glucose, lactic acid, and pyruvic acid. In the next month, while the dogs were still on the

¹ We wish to thank The Procter and Gamble Company for their generous supplies of Crisco.

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same diet, control values were also established for the effect of the injection of pyruvic acid on the levels of glucose, lactic acid, and pyruvic acid. The following method was used for all experiments. A 2 cc sample of blood was collected from the femoral artery in a 2 cc syringe. 1 cc of it was expressed directly into 5 cc of cold trichloroacetic acid for pyruvic acid analysis by the method of Friedemann and Haugen (13), the other cc was expressed into 8 cc of Somogyi's Solution I (14) to be analyzed for lactic acid by the Barker and Summerson method (15) and for glucose by the Hagedorn and Jensen method (16).

The control sample was taken after a preliminary resting period of $\frac{1}{2}$ hour. For the glucose tests, the remaining samples were drawn after 15, 30, 60, 90, and 120 minutes. After the intravenous injection of 0.5 gm per kilo of freshly distilled pyruvic acid neutralized to pH 6.3, blood samples were collected at 5, 20, and 60 minutes.

Three sets of tolerance tests were performed on each dog. The first set consisted of the control glucose and pyruvic acid tests on each of the sixteen dogs as mentioned above. After these had been completed, the ten experimental animals were placed on the vitamin B₁-deficient diet 3 to 8 weeks after vitamin B₁ had been eliminated from their diet and before they exhibited the neurological symptoms of vitamin B₁ deficiency, they were all subjected to the glucose test, and eight of them to the pyruvic acid test. Finally, the third set of tests was performed on each of seven animals as they manifested the severe acute signs of vitamin B₁ deficiency. This last set of experiments differed from the previous ones in that in six of the animals the pyruvic acid test immediately followed the glucose test, the 2 hour blood sample being the control for the pyruvic acid test. In a seventh experiment of this group, the glucose and pyruvic acid tests were made on two different animals, one received only the glucose test and the other only the pyruvic acid test, since it was deemed unwise to withhold vitamin B₁ long enough for both of these examinations.

Results

The results are presented in three groups (see Table I). The first group represents the effects of glucose and pyruvic acid injection on the blood glucose, pyruvic acid, and lactic acid for the first set of observations made on the dogs, *i. e.*, those done while the dogs were on the synthetic diet plus brewers' yeast and the full complement of vitamins. For the glucose test a ratio of approximately 7.4 is observed between lactic acid and pyruvic acid. Lactic acid rose from an average of 6.8 mg per cent to a maximum average of 10.7 mg per cent at 30 minutes and decreased to an average slightly below the control value in 2 hours. Pyruvic acid starting with an initial level of 0.9 mg per cent went to 1.3 mg per cent in 30 minutes.

and returned to its original level in 2 hours. These animals all showed a normal glucose tolerance curve. The injection of pyruvic acid into the dogs at this time did not alter the level of glucose significantly. The average concentrations of lactic acid and pyruvic acid rose, however, to 31.9

TABLE I

Glucose and Pyruvic Tests in Normal Dogs, Partially Depleted Dogs, and Dogs in Acute Vitamin B₁ Deficiency

The results are expressed in mg. per cent

No. of dogs	Condition of dogs		Glucose tolerance					
			Control	15 min	30 min	60 min	90 min	120 min
16	Normal	Glucose	83	462	264	123	95	93
		Lactic acid	6.8	10.0	10.7	7.9	7.0	6.2
		Pyruvic "	0.9	1.2	1.3	1.1	1.1	0.9
		Lactic pyruvic	7.9	8.2	8.0	6.9	6.5	6.8
10	Thiamine-deficient, no symptoms	Glucose	130	639	355	191	131	113
		Lactic acid	12.5	16.4	20.5	18.0	15.4	13.2
		Pyruvic "	2.1	2.6	2.9	2.6	2.2	2.1
		Lactic pyruvic	5.8	6.2	7.2	7.0	6.9	5.4
7	<i>In extremis</i>	Glucose	199	670	481	365	259	249
		Lactic acid	67.5	77.4	76.3	65.9	61.1	56.2
		Pyruvic "	6.3	7.0	7.0	6.5	5.9	5.7
		Lactic-pyruvic	10.7	11.0	10.8	10.2	10.4	9.8
			Pyruvic acid tolerance					
			Control	5 min	20 min	60 min		
16	Normal	Glucose	85	88	89	90		
		Lactic acid	6.8	31.9	17.2	7.4		
		Pyruvic "	0.9	16.3	2.6	1.2		
		Lactic pyruvic	7.5	1.9	6.6	6.1		
8	Thiamine-deficient, no symptoms	Glucose	157	135	124	122		
		Lactic acid	14.3	53.4	52.6	25.3		
		Pyruvic "	2.2	35.9	9.5	4.9		
		Lactic pyruvic	6.6	1.5	5.7	5.2		
7	<i>In extremis</i>	Glucose	227	208	182	156		
		Lactic acid	57.6	97.2	90.7	94.4		
		Pyruvic "	5.6	63.1	13.1	7.7		
		Lactic-pyruvic	12.8	1.5	6.9	12.8		

and 16.3 mg. per cent respectively, and decreased to 7.4 and 1.2 mg. per cent, values approaching the control values, at the end of 1 hour.

In the second set of observations, *i.e.* those made on animals which had been on a vitamin B₁-deficient diet but did not yet exhibit symptoms of deficiency, the glucose test discloses that three of the animals had high

postabsorptive glucose levels, and that 2 hours after the injection the glucose had not returned to normal levels in six dogs. Only four of the animals showed postabsorptive lactic acid values above the normal range, but after injection the lactic acid in all but two rose to abnormally high concentrations. The pyruvic acid curves of all of these animals starting postabsorptively and continuing after the injection of glucose were abnormally high. At the time the pyruvic acid test was made, four of the eight animals had high postabsorptive glucose levels. These postabsorptive values were somewhat different from those obtained at the time of the glucose test, as they were taken at a later date when the animals were in a more deficient state. In four of the animals the lactic acid concentrations in the postabsorptive resting state were still within the normal range, but after pyruvic acid injection the blood lactic acid of all but one of them rose to abnormally high levels and remained high for the duration of the experiment. The pyruvic acid values were all high.

In the third set of observations, *i.e.* those performed on animals manifesting such frank signs of vitamin B₁ deficiency as anorexia, vomiting, weakness, and especially the terminal signs of convulsions and opisthotonos, all showed abnormally high glucose, lactic acid, and pyruvic acid blood values both postabsorptively and following the injection of either glucose or pyruvic acid.

The average ratio of lactic acid to pyruvic acid in the normal dog in the postabsorptive condition is 7.9, the lowest value being 3.8 and the highest 11.6. The controls taken for the pyruvic acid test exhibited very close values to those for the controls taken in the glucose test with an average of 7.5, thus demonstrating the reproducibility of the present results. The average lactic to pyruvic acid ratio decreased a little during the glucose test, the average for all the values being 7.4. The injection of glucose, therefore, exerted little influence upon this ratio. It is obvious that in the partially deficient animals, *i.e.* those not yet showing the neurological symptoms of vitamin B₁ deficiency, a tendency to be low is observed, with an average of 6.4 for the glucose tolerance test. The lactic to pyruvic ratios for the group of animals manifesting severe acute signs of vitamin B₁ deficiency are all abnormally high and during the glucose test averaged 10.5. The ratio was decreased by the injection of pyruvic acid and although it rose rapidly towards normal during the first 20 minutes it had not in most instances reached the control value by 60 minutes.

DISCUSSION

Changes in the concentration of the three substances studied, glucose, lactic acid, and pyruvic acid, were found to vary in accordance with the severity of the deficiency. The glucose curves showed signs of disturbance

even in the group of animals which were only partially deficient. There were three instances of high postabsorptive levels and after the injection of glucose three of the curves had not returned to normal levels at the end of 3 hours. More marked is the effect observed during the acute episodes. In all but one instance, in an animal which had been vomiting repeatedly, the postabsorptive level was exaggerated, and the glucose tolerance curve was high and prolonged. These curves resemble those obtained in diabetes, in which we know carbohydrate oxidation is diminished and storage of liver glycogen is impaired. In our vitamin B₁-deficient animals similar changes may be the cause of the diabetic type of curve. These two changes may be attributed directly to lack of the vitamin, or indirectly to the influence of deficiency in the islands of Langerhans and the liver, or they may simply be due to hunger diabetes. The third possibility is considered improbable, because the animals that ate best exhibited the deficiency symptoms earliest, but nevertheless showed the same diabetic type of curve.

The cause for the accumulation of pyruvic acid and lactic acid has been established by the observations of many authors (1, 5). Since diphospho-
thiamine is necessary for the oxidation of pyruvic acid, in vitamin B₁ deficiency pyruvic acid must accumulate in the tissues and blood stream (5). Furthermore, since a ratio exists between the amount of pyruvic and lactic acids in the blood (8), it might be expected that the lactic acid concentration should also rise. Pyruvic acid is reduced to lactic acid in the blood or is absorbed by the tissues for oxidations. Previous work has shown that the ratio of lactic to pyruvic acid, even during rest, varies from species to species. Moreover, in the individual it varies still more during exercise. Friedemann and Barborka (9) find a higher ratio, with lactic acid values raised above 20 mg per cent by exercise. In early thiamine deficiency we observed a lower than normal ratio, but for animals *in extremis* the ratio was observed to attain maximal values, all but one of them above 10. The low ratio in the partially deficient animals is in accordance with the previous experiments of Stotz and Bessey (8). They observed a lower than normal ratio in thiamine-deficient pigeons and interpreted this finding as indicating a marked decrease in pyruvic acid breakdown. Similar falls may be calculated from the data reported by Williams, Mason, Power, and Wilder (17) in human beings in experimental thiamine deficiency. Such high ratios as we have observed in our animals *in extremis* have not been previously reported. The tissues of these animals are able to utilize neither pyruvic nor lactic acid adequately. The rise in lactic acid, especially, may be considered in relation to the reported inability of the liver to retain glycogen in instances of extreme vitamin B₁ deficiency (18). It is well known that acidosis causes a depletion of liver glycogen, and the high pyruvic acid and especially the high lactic acid must tend to produce an acidosis.

SUMMARY

Normal controls for the response to the injection of glucose and pyruvic acid in terms of the changes of lactic acid and pyruvic acid in the blood have been established. The postabsorptive value for the lactic acid to pyruvic acid ratio in the normal dog has been found to be 7.9 and 7.5 in two sets of experiments, each set on sixteen dogs, yielding an average of 7.7 for all our observations on normal dogs. The injection of glucose exerted little influence on this ratio and the average lactic acid to pyruvic acid ratio for the whole series of observations during the glucose tolerance test was 7.4.

As a result of vitamin B₁ deficiency in most instances, the postabsorptive level of blood sugar became high and the curve exaggerated. Both lactic acid and pyruvic acid accumulated in the blood of vitamin B₁-deficient animals. In partially deficient animals the average lactic acid to pyruvic acid ratio was lower than in the normal, with postabsorptive values of 5.8 and 6.6, *in extremis* it was higher than in the control, with a post-absorptive value of 10.7.

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THE OCCURRENCE OF SOME PREVIOUSLY UNREPORTED FATTY ACIDS IN PEANUT OIL

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One of the early investigations of peanut oil was that made by Jamieson, Baughman, and Brauns (1), who examined two types of crude oil which they had expressed from Virginia and Spanish types of peanuts, respectively. They isolated lignoceric, arachidic, stearic, and palmitic acids and identified these products by melting point determinations and elementary analyses. The presence of oleic and linoleic acids in the original oil was established by the bromine content of the respective bromine addition products of the two acids. Holde, Bleyberg, and Rabinowitsch (2), working at a later date, claimed to have isolated behenic acid from peanut oil and reported the presence of palmitic, stearic, arachidic, lignoceric, and carnaubic acids, as well. Longenecker (3), using peanut oil to test a fractionation equipment, reported the presence of myristic and palmitoleic acid in peanut oil, together with the acids noted by the previous investigators. Longenecker based the identification of the compounds which he reported on the iodine numbers and saponification equivalents of the fractions separated.

In order to determine the nature of spoilage which had occurred in a large consignment of peanut butter, a study was made of commercial peanut oil. For this purpose we purchased an edible grade of peanut oil having an iodine number of 90.7, a saponification number of 193.3, and a refractive index of 1.45, at 20°. The methyl esters from 9.5 kilos of peanut oil were prepared by refluxing in 1.5 kilo portions on a water bath 14 to 15 hours with 4 liters of methyl alcohol containing gaseous hydrogen chloride.

After the mixture of esters had been cooled and washed three times with 5 or 6 liters of water, the last traces of which were removed under reduced pressure, 8956 gm of methyl esters remained and were fractionally distilled four times at 15 mm pressure. In the first three distillations, fractionations were made at 20°, 10°, and 5° intervals, respectively, a fourth refractionation, also at 5° intervals was made to obtain a sharper separation of esters. During the first two fractionations, distillation progressed at a rate such that the drops could just be counted as they fell into the receiving flask. During the last two refractionations, the rate was about 100 drops a minute. The final distillation yielded thirty-eight ester fractions, the

first of which boiled below 85° at 15 mm pressure, and the last had not distilled at 265° under 15 mm pressure

Since we could find no report of any low boiling acids in the esters present in peanut oil, we decided to examine the material more thoroughly in order to determine whether such compounds had escaped the notice of previous workers

EXPERIMENTAL

Iodine and saponification numbers obtained for each of the thirty-seven fractions distilled from the crude esters comprise the data in Table I. The weights of the fractions plotted against fraction numbers are the basis of Fig. 1. Because the amounts of material in Fractions 23 to 28 were so great, Curve 2, in which the scale of weights is one-fiftieth that of Curve 1, was substituted in that area. A series of peaks at the maximum concentrations of the esters of the various acids present occurred at appropriate places for the esters of C_8 , C_{12} , C_{14} , C_{18} , C_{20} , and C_{22} acids.

To show the distribution of the unsaturated acids, the iodine numbers were plotted against fraction numbers (Curve 1, Fig. 2). In Curve 2, the iodine absorption values were plotted against the fraction numbers, Curve 2, including that portion of Curve 2 which would have gone off the scale, shows the iodine absorption figures reduced to one-five-hundredth of their true values. The large peak in Curve 1 occurring for Fraction 26 is very indicative of unsaturated C_{18} acids, the peak at the same point in Curve 3 strengthens this possibility.

Bromination of portions of the various fractions in diethyl ether containing a small amount of glacial acetic acid did not result in the formation of any ether-insoluble material. Unsaturated acids containing more than two double bonds are, therefore, not present in the oil.

Since only very small amounts of material were present in several of the lower fractions, an additional 15.5 kilos of peanut oil were esterified and fractionally distilled. The use of a constant pressure regulator resulted in a sharper separation of fractions, so that no material distilled between 100 – 115° .

Isolation and Identification of Fatty Acids

Saturated Acids. Caprylic Acid—The presence of caprylic acid in the fractions distilling below 100° was established as follows.

Those fractions prepared without the use of the constant pressure regulator were combined and the saturated acids separated by distillation after bromination in ether, followed by removal of excess bromine as well as the ether. The esters so obtained distilled at 83° under 15 mm pressure. This is the boiling point for methyl caprylate as found by Lewko

witsch (4) and Jamieson (5) Those fractions prepared with the constant pressure regulator were combined and subjected to the lead-soap-ether method for removing unsaturated material A saturated acid thus iso-

TABLE I
Some Constants of Fractions Made at 5° Intervals (15 Mm Pressure) of Methyl Esters from Peanut Oil

Fraction No	B p 15 mm	Weight	I No	Iodine absorption per fraction	Mean mol wt of acids
		gm		gm	
1	85	16.7	1.7	0.2839	141.3
2	85-90	14.5	1.3	0.1885	147.9
3	90-95	6.5	2.1	0.1365	150.1
4	95-100	2.0	*	*	*
5	100-105	2.5	*	*	*
6	105-110	1.2	*	*	*
7	110-115	1.7	*	*	*
8	115-120	7.0	1.5	0.105	176.6
9	120-125	10.5	1.9	0.1995	180.5
10	125-130	13.5	2.5	0.3375	185.4
11	130-135	22.0	1.5	0.360	193.1
12	135-140	69.0	1.7	1.173	200.8
13	140-145	67.5	2.4	1.62	201.0
14	145-150	54.5	2.5	2.725	202.8
15	150-155	46.0	2.7	0.702	209.6
16	155-160	16.0	3.5	0.175	209.7
17	160-165	5.0	4.1	0.08	210.4
18	165-170	17.5	17.1	1.2425	219.1
19	170-175	14.1	23.0	3.243	224.5
20	175-180	18.0	29.1	5.238	234.6
21	180-185	24.7	37.0	9.139	237.3
22	185-190	53.5	35.8	20.153	244.3
23	190-195	158.0	43.4	68.572	252.2
24	195-200	396.0	61.1	241.956	262.7
25	200-205	1657.5	83.2	1379.04	271.3
26	205-210	4107.7	106.3	4366.4851	277.5
27	210-215	870.8	103.5	901.278	277.9
28	215-220	107.4	91.4	98.1636	279.6
29	220-225	48.2	80.5	38.801	287.8
30	225-230	31.0	70.7	21.917	294.0
31	230-235	15.2	56.0	8.512	301.1
32	235-240	17.0	43.6	7.412	304.6
33	240-245	29.2	31.5	9.198	317.0
34	245-250	43.0	22.6	9.71	322.3
35	250-255	29.8	11.9	3.55	339.3
36	255-260	61.8	6.2	3.83	341.0
37	260-265	32.5	4.9	1.59	354.6
38	*	*	*	*	*

* Not determined

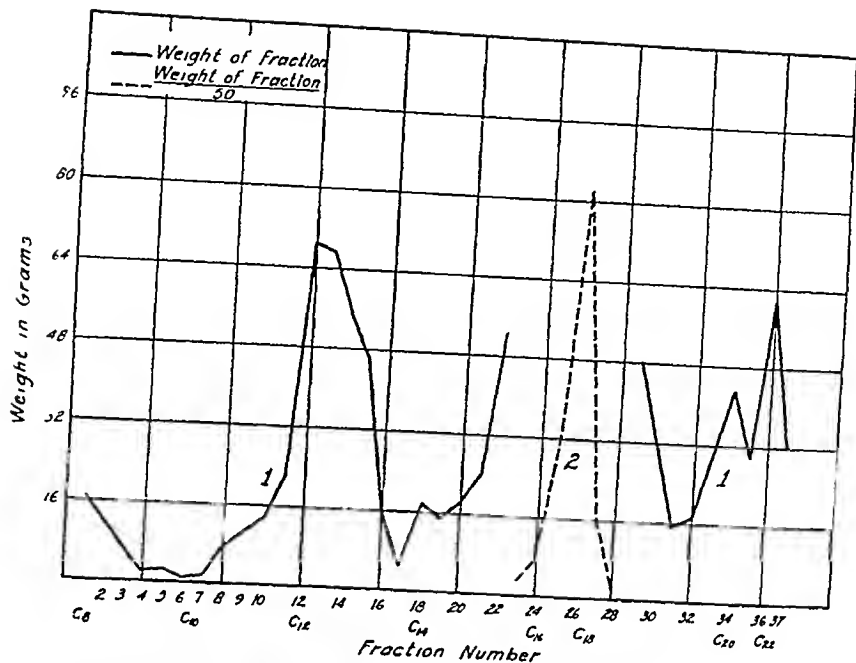


FIG 1 Curves derived from the weights of the several fractions

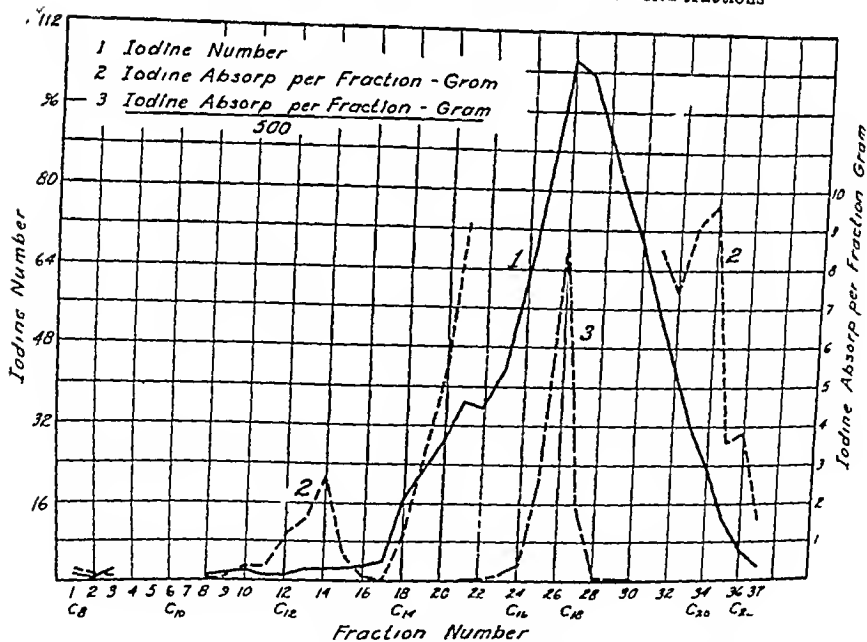


FIG 2 Curves derived from the iodine numbers of the several fractions

lated, after several recrystallizations from 95 per cent ethyl alcohol, possessed the following constants melting point 15.7° , melting point of caprylic acid 16.5° , molecular weight 147.6, molecular weight of caprylic acid 144.12

Lauric Acid—Although the absence of capric acid seemed probable from the appearance of the curve (Fig 1), nevertheless, the fractions boiling between $115\text{--}130^{\circ}$ were investigated for the presence of this acid. The saturated esters were separated from unsaturated material by bromination of the combined fractions and distillation as in the case of the acids previously described. The saturated material thus separated possessed the characteristics shown in Table II.

Inspection of the data for the material boiling below 125° reveals that the melting points are too low and the molecular weights too high for capric acid. The melting point and molecular weight of the material distilling between $125\text{--}130^{\circ}$ agree closely with the values for lauric acid. It is, therefore, probable that the small amount of material originally

TABLE II
Identification of Lauric Acid

Observed for acid isolated	Fraction boiling (15 mm pressure) at			Accepted values	
	115-120	120-125	125-130	Capric acid	Lauric acid
M p, $^{\circ}\text{C}$	16.0	26.5	43.4	31.3	45.6
Mol wt	176.1	191.6	199.01	172.15	200.19

distilling between $100\text{--}125^{\circ}$ at 15 mm consisted of a mixture of C_8 and C_{12} saturated acids.

The presence of lauric acid previously suspected in Fractions 12 to 15, because of the characteristic peak in the curve (Fig 1), was definitely established by isolating the free acid from the esters and recrystallizing several times from 95 per cent alcohol. The acid so prepared possessed the following constants: observed melting point 43.5° , melting point of lauric acid 43.6° , observed molecular weight 201.9, molecular weight of lauric acid 200.19, iodine number 0.8, iodine number of lauric acid 0.0.

Myristic Acid—The presence of myristic acid in the fractions boiling between $155\text{--}175^{\circ}$ was suspected from the appearance of the curve (Fig 1), and was established in the following manner. The saturated material was separated from any unsaturated compounds present by the lead-soap-ether method. The acid isolated from the ether-insoluble lead-soap possessed the following constants when recrystallized several times from 95 per cent alcohol: observed melting point of the lead salt 108.0° , melting point of lead myristate 107.0° , observed melting point of the acid 53.5° ,

melting point of myristic acid 54.0°, observed molecular weight of the acid 228.9, molecular weight of myristic acid 228.2, observed iodine number 15, iodine number of myristic acid 0.0

Palmitic Acid—Palmitic acid was isolated from the combined methyl esters boiling between 175–190° as well as from those boiling between 190–195° and 195–200° by the lead-soap-ether separation. The fatty acid so obtained possessed the constants shown in Table III after several recrystallizations from 95 per cent alcohol.

TABLE III
Identification of Palmitic Acid

	Fraction boiling at			Accepted values for palmitic acid
	145–190°	190–195°	195–200°	
M p of lead salt, °C	114	110–111	110	112
" " acid, °C	62	62.1	61.9	62.6–63
Mol wt of acid	257.2	256.4	257.4	256.3
I No of acid	1.89	1.07	3.75	0.0

TABLE IV
Identification of Stearic Acid

	Fraction boiling (15 mm pressure) at			Accepted values for stearic acid
	200–205°	205–210°	210–215°	
M p of lead salt, °C	118			115.6
" " acid, °C	65	67	62.1	69.6
Mol wt of acid	279.9	284.4	292	284.3
I No of acid	1.6	0.45	4.2	0

Undoubtedly, the peak for the C₁₈ saturated acid occurs in the fraction boiling between 190–195°, for it is in this fraction that the closest agreement occurs. The saturated acid from the fraction boiling between 195–200° probably contains small traces of stearic acid.

Stearic Acid—Stearic acid was present in several of the fractions but the best preparation was isolated by the lead-soap-ether method from the methyl esters boiling between 205–210° at 15 mm. The fatty acids so obtained (or their derivatives) possessed the constants given in Table IV after several recrystallizations from 95 per cent alcohol.

Arachidic Acid—The presence of arachidic acid was definitely established in the fraction distilling between 215–220° at 15 mm after separation by the lead soap-ether method and also after low temperature crystallization of the methyl esters from acetone. Although stearic acid was present in

the same fraction, the arachidic acid crystallized first from ethyl alcohol. The best arachidic acid preparation was that obtained by redistilling, at 5 mm pressure, portions of the material boiling above 230° at 15 mm. The fraction boiling at 205–210° at 5 mm pressure, when subjected to low temperature crystallization, yielded arachidic acid of high purity. Constants for the acid obtained from these fractions are given in Table V.

Behenic Acid—Behenic acid was identified definitely in the methyl esters distilling between 245–250° and 250–255° at 15 mm. It is probable that the mixture distilling between 240–245° at 15 mm contained a mixture of arachidic and behenic esters, since after repeated attempts to purify the material by distillation at 0.3 mm pressure, a melting point of 77.5° and

TABLE V
Identification of Arachidic Acid

	Fraction boiling at 215–220 15 mm	Fraction boiling at 205–210 5 mm	Accepted values for arachidic acid
M p of acid, °C	74	76–77	77
" " methyl ester, °C	47.5	46.5	46.5–47.5
Mol wt of acid	301.8	309.3	312
I No of acid	5.21	1.9	0

TABLE VI
Identification of Behenic Acid

	Fraction boiling at				Accepted values for behenic acid
	240–245 15 mm	245–250° 15 mm	250–255 15 mm	210–220 5 mm	
M p of acid, °C	77.5	79	79.5	79.1	80.2
" " ester, °C	49.5–50	49.7	50	49.6–50	50
Mol wt of acid	328	336	339	339.2	340
I No of acid	7.8	0.9	0.5	2.47	0.0

a molecular weight of 328 were obtained for the acid. The purest acid was prepared from the fraction distilling between 210–220° at 5 mm (obtained by redistilling at 5 mm pressure portions of the material boiling above 230° at 15 mm). Constants for the acids obtained from these fractions are given in Table VI.

Lignoceric Acid—Lignoceric acid was probably present to a slight extent in several fractions. The best preparation was obtained from the esters distilling between 260–265°, at 5 mm pressure. The material was fractionally crystallized from 95 per cent alcohol and again recrystallized several times after the acid had been freed from the esters. The constants obtained for these acids are given in Table VII.

Unsaturated Acids 9-10 Oleic Acid—9-10 oleic acid was identified in several of the fractions beginning with the methyl esters distilling between 190-195° at 15 mm pressure. Two general methods were used for the isolation of oleic acid (a) separation by the lead-soap-ether method, followed by further purification by the barium-soap-benzene process, (b)

TABLE VII
Identification of Lignoceric Acid

	Fraction boiling at 260-265° 15 mm	Fraction boiling at 220-230 5 mm	Accepted values for lignoceric acid
Mol wt	365	367	368.4
M p of acid, °C	83.1	82.9	84.2
" " methyl esters, °C	55	54.5	56.7
I No	0.49	1.95	0

TABLE VIII
Constants of Oleic Acid and Derivatives Isolated from Various Fractions

	Acid isolated from fraction boiling (15 mm pressure) at					Corresponding values for oleic acid
	190-195	195-200	200-205°	205-210	210-215	
Mol wt of acid	283.5	279.5	284.7	282.29	266.03	282.0
I No of acid	88.45	87.04	93.3	102.6	83.47	89.93
M p of dihydroxy derivative			128	128.6	128.8	130
Mol wt of dihydroxy derivative			314.7	315.1	315.2	316.0

TABLE IX
Constants of Linoleic Acid and Derivatives Isolated from Various Fractions

	Acid isolated from fraction boiling (15 mm pressure) at						Corresponding values for linoleic acid
	200-205	205-210	210-215	215-220°	220-225	225-230	
Mol wt of acid	289.9		243	292.5	273.5	277	282.1
I No of acid	154.7	141.2	141.1	140.2	112.8	113.2	181
M p of tetrabromide, °C	113	111	113.5		113.0	113.5	114

low temperature crystallization of the methyl esters from an 8 per cent solution in acetone. Identification was made by means of the molecular weight, iodine number, and preparation of the crystalline dihydroxystearic acid by oxidation with alkaline permanganate solution (Table VIII).

Linoleic Acid—Linoleic acid was identified in several fractions beginning

with the methyl esters distilling between 200–205° at 15 mm pressure. Two general methods were used for the isolation of the linoleic acid (a) separation by the barium-soap-ether method, (b) low temperature crystallization of the methyl esters from an 8 per cent solution in acetone. Identification was made by preparation of the crystalline tetrabromide, since in no case was it possible to isolate a pure preparation of the linoleic acid (Table IX).

Although slight peaks occurred in the iodine absorption curve (Fig. 2) at the ranges 145–150° and 245–250° at 15 mm, we were unable to isolate additional unsaturated fatty acids from this material. The fractions boiling between 175–200° at 15 mm were carefully examined for the presence of palmitoleic acid previously reported by Longenecker (3). However, a redistillation of this material at 0.3 mm pressure yielded oleic acid as the only unsaturated material present.

SUMMARY

- 1 Caprylic and lauric acids were isolated for the first time from peanut oil.
- 2 The presence of myristic, palmitic, stearic, arachidic, behenic, lignoceric, oleic, and linoleic acids, previously reported as occurring in peanut oil, was confirmed.
- 3 The oleic acid found was the 9–10 isomer.

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AMINO ACID ABSORPTION AND UTILIZATION IN THE CHICK

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The rates of absorption of amino acids from the gastrointestinal tract have been reported for the rat (1) and the levels of blood amino nitrogen during amino acid absorption have been reported for the rat, dog, and rabbit (2-7), but no studies of this nature have been reported for the chick. In view of the known differences between avian and mammalian nitrogen metabolism (8), it seemed desirable to study amino acid absorption and utilization in the chick. It is the purpose of this paper to present data concerning amino acid absorption in the chick and changes in blood amino nitrogen during the amino acid absorption. These data are compared with those for mammals which have been reported in the literature.

EXPERIMENTAL

The rates of absorption of amino acids from the gastrointestinal tract of the chick were determined by the Cori technique (9, 10). All of the experiments were conducted with single comb white Leghorn chicks which had been previously fed a normal chick diet. The chicks were fasted for 48 hours before the amino acid administration, but were allowed water *ad libitum*. The majority of the chicks weighed between 150 and 250 gm at the end of the 48 hour fast.

The amino acids¹ used were commercial preparations. With the exception of tyrosine, all of the amino acids were administered in solutions containing 12 to 31 mg of amino nitrogen per cc. To establish uniform conditions, an attempt was made to administer the amino acids after half neutralization with sodium hydroxide. When this was impossible, the amino acid was administered in a more soluble form, either as the complete sodium salt, or as the hydrochloride. Tyrosine, which was not soluble enough to give the desired concentration even as the sodium salt, was administered as a well shaken suspension. The concentration of each solution was determined by both micro-Kjeldahl and amino nitrogen analyses. The amino acids in solution were administered directly into the crop by means of a calibrated syringe to which a piece of No. 6 catheter tubing was

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¹ The amino acids were purchased with funds donated by the Nutrition Foundation, Inc.

attached The tyrosine suspension was administered to the crop by means of a serological pipette which was washed out after the administration to determine the tyrosine remaining on it During the absorption period, the vent was clamped with a paper clamp (Hunt clip No 0) to prevent any excretion

To determine whether material injected into the crop passed quickly into the absorbing portions of the gastrointestinal tract, a solution of methylene blue was administered to two fasted chicks The first chick was killed after 5 minutes and the methylene blue was found in the crop, proventriculus, and gizzard The second chick was killed after 10 minutes and the methylene blue was found as far as half-way along the small intestine Henry *et al* (11) found that in a fasted chick the crop discharges material into the gizzard almost immediately

All values of the absorption rate were obtained for a 3 hour absorption period At the end of 3 hours the chick was killed by dislocating the neck, and the esophagus was tied near the pharynx The entire gastrointestinal tract was then removed and frozen in a refrigerator Upon removal from the refrigerator, the exterior of the gastrointestinal tract was carefully washed with warm water and the mesentery was removed The tract was then opened, washed four times with distilled water, and the volume made up to 500 cc An aliquot of this solution was diluted with an equal volume of 10 per cent trichloroacetic acid to precipitate the proteins, which were then removed by filtration

Amino nitrogen was determined in the filtrate by the method of Folin (12) as modified by Danielson (13) The method was further modified by making all of the determinations in 1.66 per cent trichloroacetic acid Before procedure with the standard method, each sample was neutralized to the phenolphthalein end-point with sodium hydroxide All of the readings were made between 20 and 22 hours after addition of the sodium β -naphthoquinone-4-sulfonate reagent and 8 minutes after the bleaching reagents A Cenco photometer with Cenco Filter 2 (green) was used for making the readings A standard curve with the amino nitrogen concentration plotted against its equivalent photometer reading was used in converting the photometer reading to amino nitrogen values

Weighed samples of the amino acids used in the study were analyzed by both micro-Kjeldahl and amino nitrogen methods The values obtained for amino nitrogen were used in calculating the absorption rate in terms of the particular amino acid studied

To eliminate the turbidity which occurred when large amounts of tryptophane were present, the solutions were filtered just before the photometer readings were made, and a separate standard curve was prepared for tryptophane with filtered solutions The amino nitrogen of the contents

of the gastrointestinal tract of chicks with and without the administration of tryptophane was calculated as tryptophane from the standard curve for this amino acid. The tryptophane absorption rate was determined for three chicks by this modification of the Folin method.

In order to find the normal amino nitrogen content of the gastrointestinal tract of fasted chicks, distilled water instead of amino acid solution was given to twenty-three chicks during the course of the study. The chicks were killed 3 hours after the administration of 1 cc of water per 100 gm of body weight. The residual amino nitrogen values varied between 4.66 and 10.04 mg per 100 gm of body weight. The mean value of 7.43 ± 1.65 mg of amino nitrogen per 100 gm of body weight was used for all calculations of absorption rate.

To compare the amino nitrogen method of determining the amino acid with a specific amino acid method, the absorption rate of methionine was determined by measuring the unabsorbed methionine by the amino nitrogen method previously described, and by the specific methionine method of McCarthy and Sullivan (14). Methionine determinations on the contents of the gastrointestinal tract of fasted chicks showed that no measurable amount of methionine was present. The specific methionine method eliminates the use of a figure for residual amino nitrogen and should give a truer absorption value for an individual chick. The average methionine absorption value obtained by the amino nitrogen method was 45.6 mg per 100 gm of body weight per hour, compared with the value of 42.6 mg obtained by the specific methionine method. The rate of absorption, even as determined by the specific methionine method, showed considerable variation among chicks. For the whole series, the two methods of determining the rate of methionine absorption showed satisfactory agreement.

Blood samples were obtained from chicks immediately before and 1 hour after the administration of amino acid. 1 cc samples were taken by heart puncture, with potassium oxalate as an anticoagulant, and blood filtrates were prepared with trichloroacetic acid as a precipitant. Amino nitrogen was determined on these blood filtrates by the same method as was employed on the gastrointestinal tract contents. Taking both initial and 1 hour blood samples from each chick enabled a more accurate comparison of changes in the blood amino nitrogen after the administration of different amino acids than would have been possible if only one sample had been taken. A preliminary experiment showed 1 hour to be the time interval during which blood amino nitrogen increased most after amino acid administration. Initial and 1 hour blood samples were also taken from the chicks to which water was administered. The 1 hour value varied between 79 and 101 per cent of the initial value, and its mean was 88.6 ± 5.5 per cent of the initial value.

Intact protein was studied from the standpoint of changes in the blood amino nitrogen over a 5 hour period. The effect of hydrolysis of protein was investigated by comparing solutions of intact casein and gelatin with

TABLE I

Rates of Absorption of Amino Acids in Chick, and Blood Amino Nitrogen Level at 1 Hour after Amino Acid Administration

Amino acid	Salt	No of chicks	Absorption rate (quantity per 100 gm body weight per hr)			Blood amino N as per cent of initial	Concentration of solution administered
			Amino N (1)	Amino acid			
				(2)	(3)		
			mg	mg	mm		mg amino N per cc
dl-Alanine	Half Na	7	6.82	43.3 ± 9.9	0.486	121 ± 12.4	31
l(+)-Arginine	HCl*	8	1.54	27.0 ± 7.5	0.155	95 ± 5.8	12
"	"	7	2.15	37.6 ± 7.5	0.216	95 ± 7.1	30
l(+)-Aspartic acid	Half Na	7	2.92	40.8 ± 5.9	0.306	93 ± 6.0	21
l(-)-Cystine	Na	12	5.02	47.6 ± 12.6	0.198	101 ± 4.3	26
l(+)-Glutamic acid	Half Na	9	3.58	45.5 ± 12.3	0.309	96 ± 7.4	27
" "	" "	6	2.44	30.9 ± 7.6	0.210	95 ± 6.2	39
Glycine	" "	7	7.33	39.0 ± 7.2	0.520	129 ± 8.9	30
"	" "	7	7.38	39.3 ± 6.0	0.524	121 ± 19.3	29
l(+)-Histidine	HCl	10	2.51	42.3 ± 14.1	0.273	103 ± 8.8	20
l(-)-Leucine	Na	5	3.64	37.2 ± 2.6	0.284	118 ± 4.6	22
dl-Lysine	Half Na	6	4.95	23.7 ± 1.2	0.162	111 ± 7.6	30
dl-Methionine	Na	7	3.82	45.6 ± 11.5	0.306	120 ± 11.2	22
dl-Phenylalanine	"	7	4.72	56.2 ± 9.6	0.340	102 ± 4.9	24
l(-)-Proline	Half Na	10	5.50	49.8 ± 9.7	0.433	110 ± 14.8	25
dl-Threonine	" "	9	4.05	36.5 ± 11.3	0.306	109 ± 9.6	29
dl-Tryptophane	Na	3	1.49	21.8 ± 16.5	0.107	95 ± 3.2	20
l(-)-Tyrosine	"	6	2.50	36.3 ± 17.2	0.200	93 ± 6.3	14
"	"	6	3.79	54.9 ± 12.9	0.303	88 ± 9.7	20
Glycine + dl-alanine	Half Na	7	7.42	43.1 ± 7.9	0.525	117 ± 12.9	31
Casein hydrolysate		6	7.36	66.6 ± 5.0	0.462	108 ± 9.6	26

* Monohydrochloride

acid hydrolysates of these proteins. The method of Caldwell and Rose (15) was used in preparing the protein hydrolysates.

Results

The data obtained for the absorption rates and blood amino nitrogen for the amino acids and amino acid mixtures used are summarized in Table I. The absorption rate for amino acid (Column 1) is the value obtained

in terms of amino nitrogen by the modified Folin method. Column 2 shows the absorption rate for the free amino acid, calculated from Column 1 and the correction for the particular amino acid. In Column 3 the absorption rate is expressed as mm of the amino acid.

The data indicate that all of the amino acids apparently were readily absorbed from the gastrointestinal tract. The variation among individual chicks in the study of a single amino acid was appreciable in many cases, hence close comparison of the absorption rates is not justified. In an attempt to determine whether the level of amino acid administration affected the rate at which it was absorbed, glycine, glutamic acid, tyrosine, and arginine were administered at two levels. Glycine gave practically the same absorption rate at a high level of administration as it did at the usual level. Tyrosine and arginine gave higher absorption values with larger amounts administered, but glutamic acid gave a lower value. The variability was high in all cases and it is doubtful whether any significance can be attached to these comparisons.

To facilitate the comparison of the absorption rate of one amino acid with others, the millimolar absorption rate was plotted against the apparent molal volume. The values of apparent molal volume used were taken from the report of Cohn *et al* (16), their observed values being used when given, when these were not available, the volumes were calculated according to their methods. When the amino acids were administered at more than one level, data for the level nearest 30 mg of amino nitrogen per cc were used. The plot of these data (Fig 1) shows a general tendency for the molal absorption rate of the amino acid to increase as its apparent molal volume decreases. The correlation coefficient of this relationship is -0.81 ± 0.092 , which indicates that the relationship is significant.

The level of blood amino nitrogen found 1 hour after amino acid administration is dependent upon the amount of amino acid in the blood and the ability of the particular amino acid to give color with the Folin reagent. After water administration, the blood amino nitrogen is 89 per cent of the initial value, hence subtracting 89 from the percentage values for the amino acids (Table I) gives the relative increase of blood amino nitrogen caused by the amino acid studied. When this value is expressed as weight of the amino acid and divided by the molecular weight, the relative molar increase of the specific amino acid in the blood stream is obtained. In Fig 2, this amino acid increase in the blood is plotted against the millimolar absorption rate from the gastrointestinal tract.

If all amino acids were removed from the blood stream at the same rate, it would be expected that a direct relationship would exist between the relative molar blood increase and the millimolar rate of absorption of the amino acid. The deviations from the direct relationship indicate that some amino acids are removed from the blood stream more rapidly than others.

From Fig 2 it appears that glutamic acid, aspartic acid, tyrosine, and phenylalanine are rapidly removed from the blood stream, while methionine and leucine are removed from the blood stream more slowly than the other amino acids studied

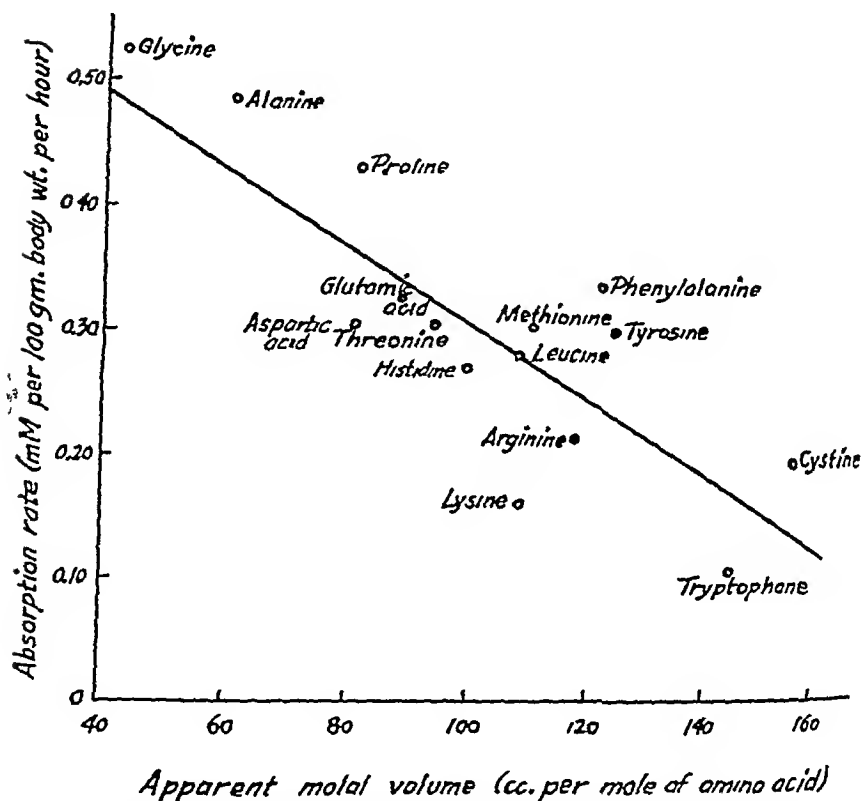


FIG 1 The relation between the millimolar absorption rate of an amino acid from the gastrointestinal tract of the chick and the apparent molal volume of the amino acid

The data for blood amino nitrogen for the studies with proteins and protein hydrolysates are summarized by Fig 3. The blood amino nitrogen reached a higher level after the administration of a protein hydrolysate than after intact protein. Gelatin caused a greater increase in blood amino nitrogen than casein when the intact and hydrolyzed forms of the two proteins were compared.

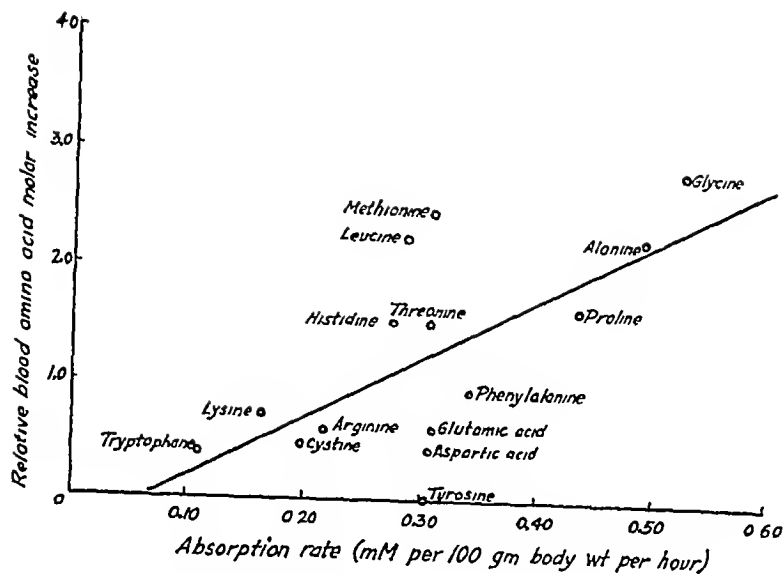


FIG 2 The relation between the relative molar increase of an amino acid in the blood and the millimolar rate of absorption of the amino acid from the gastrointestinal tract of the chick

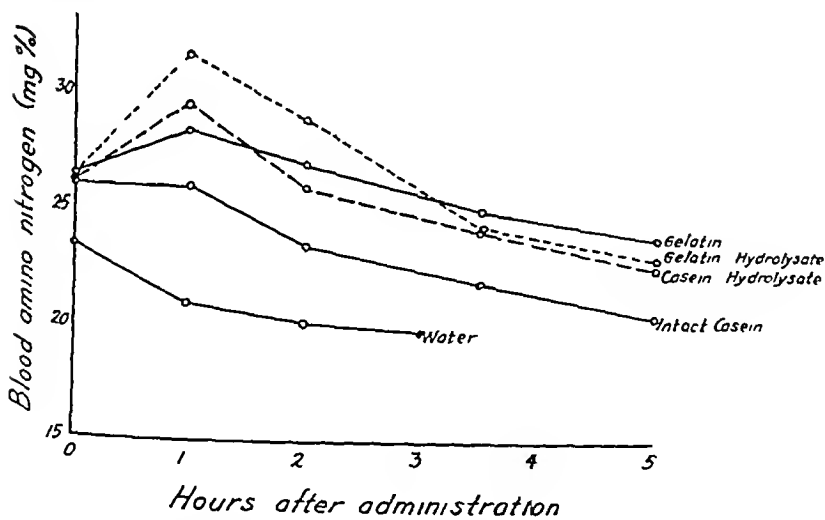


FIG 3 Curves showing the blood amino nitrogen level in chicks after the oral administration of gelatin, gelatin hydrolysate, casein, casein hydrolysate, and water. Each plotted point represents an average value for three chicks for casein, casein hydrolysate, and gelatin hydrolysate, seven chicks for gelatin, and six chicks for water.

DISCUSSION

As a basis for discussion of the blood amino nitrogen for the chick, any 1 hour value which is greater than 89 per cent of the initial value is considered an increase in blood amino nitrogen. Water administration gave 1 hour values which were 89 per cent of the initial value.

In chicks, as in dogs, rabbits, and rats (2-4), glycine and alanine caused the greatest increase (129 or 121 per cent and 121 per cent respectively of the initial) in the blood amino nitrogen after oral administration. Leucine, which was reported by Seth and Luck (2) to give a moderate increase in rabbits, caused a relatively great increase (118 per cent of the initial) in the blood amino nitrogen of chicks. Histidine caused a moderate increase in the rabbit (2) and hexone bases caused a moderate increase in rats (4). Histidine also caused a moderate increase (103 per cent of the initial) in the chick. Aspartic acid and glutamic acid (2, 3) gave slight increases in the blood amino nitrogen in dogs and rabbits, and similar results (93 and 95 or 96 per cent respectively of the initial) were obtained with chicks.

In the rabbit, the blood amino nitrogen increased only slightly after phenylalanine and tyrosine administration (5). In this respect these amino acids more closely resemble the dicarboxylic acids than glycine and alanine. A greater increase was observed after phenylalanine than after tyrosine administration. Similar differences are indicated in the chick (102 and 88 or 93 per cent respectively of the initial).

In the rabbit, the blood amino nitrogen increased moderately after lysine ingestion (3) and in the dog (6) the "blood amino nitrogen increased markedly and remained high for some time". Lysine caused a moderate increase (111 per cent of the initial) in the blood amino nitrogen in chicks.

Seth and Luck (2) obtained only a slight increase in the blood amino nitrogen level after cystine administration to dogs and rabbits. Lewis and Brown (7) reported that the cystine content of plasma increased "greatly" after cystine administration to rabbits, but no values were given to indicate the extent of the increase. Chicks show a slight (101 per cent of the initial) increase in blood amino nitrogen after cystine ingestion.

Seth and Luck (2), in one experiment, obtained practically no increase in the blood amino nitrogen of the rabbit after tryptophane administration. The chick shows a slight increase with tryptophane (95 per cent of the initial).

Despite the known differences in nitrogen metabolism which exist between the chick and other species, no marked differences between species may be seen in a comparison of the blood amino nitrogen levels after various amino acids are administered orally.

The comparisons made above between the levels of amino nitrogen in chick and mammalian blood after the ingestion of various amino acids are

of value only in comparing the metabolism of various species. To make the level of blood amino nitrogen a better index of the ability of the animal to utilize an amino acid, the amino nitrogen level may be calculated as the amino acid and may be compared with the absorption rate (Fig 2). The fact that the correlation of molar increase of blood amino acid and molar absorption rate from the gastrointestinal tract is of border line significance indicates that there are differences in the rates of removal of amino acids from the blood stream. Methionine and leucine appear to be removed from the blood stream more slowly than the remainder of the amino acids. Tyrosine, phenylalanine, aspartic acid, and glutamic acid seem to be rapidly removed from the blood stream.

The absorption values for alanine, leucine, lysine, and methionine in the chick are very similar to comparable values for the rat reported in the literature (9, 17-19). The cystine absorption value for the chick compares more favorably with the value for the rat reported by Sullivan and Hess (20) than with the value reported by Wilson (21). Glutamic acid and histidine values for the chick are considerably lower than those reported for the rat (17, 19), while glycine is slightly lower (9, 17, 22). Although the tryptophane absorption value for the chick is inconclusive, it is greatly below the value of 62.9 mg per 100 gm of body weight per hour as reported by Berg and Bauguess (23).

The fact that phlorhizin inhibits carbohydrate absorption and does not inhibit amino acid absorption (22) suggests that different mechanisms are involved. Carbohydrate absorption probably proceeds after preliminary phosphorylation, a process which can be inhibited by phlorhizin (24). Hober (25) concluded that amino acid absorption is too fast to be explained by diffusion. Hober and Hober (26) suggested that a cellular mechanism is involved in the preferential absorption of amino acids. Amino acids exhibited irregular behavior and there seemed to be no relation between size of molecule and the rate of absorption. However, this work with rat intestinal loops did not compare amino acids among themselves. Bolton and Wright (27) analyzed blood and lymph from various vessels in the cat and found that absorption of amino acids from the intestine follows the law of diffusion. Lathe (28) concluded from his work with intestinal loops in dogs that the rate of intestinal absorption of amino acids decreases with an increase in their molecular size.

Mehl and Schmidt (29) found that the diffusion coefficient of amino acids in aqueous solution is related to the size and shape of their molecules, rather than to their molecular weights. As was shown in Fig 1, the rate of absorption of an amino acid from the intestinal tract of the chick varies inversely with the apparent molal volume of the amino acid. These data would indicate that amino acid absorption in the chick is a function of rate

of diffusion of the amino acid and is not controlled by any cellular mechanism. If different amino acids were absorbed by different mechanisms, a considerable increase in the absorption rate would be expected when mixtures of amino acids were administered. Such was not the case with a mixture of glycine and alanine. The casein hydrolysate was absorbed only slightly faster than single amino acids.

Hydrolysis of protein before oral administration apparently allows a more rapid absorption of the constituent amino acids. The possibility exists that absorption of amino acid complexes takes place after protein administration, and that these complexes are relatively poorly detected by the blood amino nitrogen method. The fact that gelatin caused a greater rise in blood amino nitrogen than did casein may be due to the differences in amino acid composition. Gelatin contains more glycine, alanine, and hydroxyproline and less tyrosine, glutamic acid, and hydroxyglutamic acid than does casein (30). Glycine and alanine have been shown to cause large increases in blood amino nitrogen, while tyrosine and glutamic acid cause small or no increases in blood amino nitrogen.

SUMMARY

1 The rate of absorption of amino acids from the gastrointestinal tract of a chick varies inversely with the apparent molal volume of the amino acid.

2 In general, the blood amino nitrogen of a chick increases after the absorption of amino acids from the gastrointestinal tract in proportion to the amount of amino nitrogen absorbed. Methionine and leucine caused disproportionately high levels of blood amino nitrogen, while aspartic acid, glutamic acid, tyrosine, and phenylalanine caused disproportionately low levels of blood amino nitrogen after their oral administration.

3 The relative increase in blood amino nitrogen of a chick after the oral administration of various amino acids is similar to that observed in mammals.

4 The oral administration of a hydrolyzed protein causes a greater increase in the blood amino nitrogen of a chick than does the administration of intact protein.

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AN IMPROVED SHORT TIME TURBIDIMETRIC ASSAY FOR PENICILLIN

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As the production of penicillin has advanced from the pilot plant stage to full scale production, the need for an accurate short time test has grown more and more pressing. Such a procedure is needed not only for following the penicillin content of the fermentation tanks but also in subsequent purification steps.

In 1942 Foster (1) published a note on a 4 hour test, but from a more recent report (2) the method seems to have been too laborious and not sufficiently accurate.

It was felt that the turbidimetric method offered the best possibilities for accuracy and speed, and it was investigated thoroughly. The method presented in this paper proved not only rapid (3 to 4 hours incubation) but very precise as well. The procedure is very much like that used in the many microbiological vitamin assays. Since many people are familiar with the turbidimetric vitamin assays, they can run this penicillin assay with a minimum of instruction compared to other assay procedures, such as the Oxford plate method.

EXPERIMENTAL

Culture and Inoculum—Stock cultures of *Staphylococcus aureus* Strain H are carried on plain nutrient agar slants. Duplicate tubes of the organism are incubated at 37° for 24 hours and then held in the refrigerator. One culture is used for preparing inocula from day to day. At the end of the week, and at weekly intervals thereafter, two more slants are prepared from the unused culture. The new cultures are checked by a Gram stain to see whether they are pure, the old cultures are discarded, and the new cultures used as before.

Inoculum for the test is made by transferring a bit of the surface growth from the slant to one or more flasks containing 100 ml of plain nutrient broth (8 gm of Difco Bacto dehydrated powder per liter of water). The inoculated medium is incubated until the next day, when it is used in the test. The incubation time is usually from 14 to 18 hours.

Basal Medium—The medium being used at present has the following composition per liter: Difco Bacto-nutrient broth 16 gm, yeast extract 4 gm. This medium is used instead of regular plain nutrient broth, because

it was found to give a larger spread of the points on the standard curve. No glucose is used in the medium at present owing to the unfavorable report presented by Dr Foster (3). 0.5 per cent glucose increases the speed of growth very greatly and spreads out the curve. In a preliminary test the shape of the curve remained the same and the value obtained on a fermentation liquor was essentially the same as by the regular method. Further tests are being run and glucose may be added to the medium later if the tests show it to be desirable.

Varying amounts of the basal medium (depending on the number of tubes being used per test) are put in flasks, stoppered with cotton plugs, and autoclaved at 15 pounds for 15 to 20 minutes. The flasks are then cooled and put away for use as needed.

Standard Solution—The standard solution is made up each day from a dry calcium salt of penicillin (potency = 333 Oxford units per mg) which is stored in the refrigerator at about 3–5°. About 30 mg of the standard are weighed out and taken up in 200 ml of 0.1 per cent phosphate buffer solution, pH 6.8. This solution is filtered through a micro Sartz filter, 1 ml is placed in a sterile test-tube, and sufficient buffer is added with a pipette to give a solution containing 5.00 Oxford units per ml.

Samples—These are usually filtered through a Sartz filter, although as in other short incubation tests they may be made up merely with sterile buffer unless known to be heavily contaminated. Ordinarily, the approximate potency of the material to be tested is known, and a final solution for the test may be prepared that will have a potency of from 2 to 5 Oxford units per ml.

Equipment and Procedure—The test is set up in stainless steel racks holding forty $\frac{5}{8} \times 4$ inch Kimble glass tubes covered with an inverted stainless steel tray $\frac{1}{2}$ inch deep to keep out dust contamination. Pipetting of standards and samples is done with a special pipette prepared from standard 0.2 ml Pyrex pipettes.¹

The micro pipettes are used because the small volume employed (0 to 0.16 ml) makes an insignificant difference in the final volume of medium in the tube (7 ml) and hence no buffer need be added to bring all the samples to a definite volume.

¹ A small bubble about twice the diameter of the original capillary is blown near the tip of the pipette. This is then drawn out to form a needle-like tip about 30 mm long. The top of the pipette is cut off about 40 mm above the top graduation and polished. The pipettes are operated by a 1 ml hypodermic syringe of the insulin type, attached to the pipette by a 50 mm piece of pressure tubing slipped part way over the barrel of the syringe. A thin coating of light petroleum jelly on the plunger insures smooth operation. With a little practice volumes of 0.02 to 0.2 ml can be delivered accurately and rapidly with this equipment. Caution—do not allow water to get up into the barrel of the syringe, as it will prevent smooth operation of the plunger. A small cotton plug in the rubber tube will help prevent this.

If it is desired, a fixed sample volume may be selected at say 1 or 2 ml so that 1 or 2 ml pipettes may be used. If this is done, all samples added must be made up to the selected volume by addition of buffer solution and appropriate increase made in the concentration of the basal medium. It has been the author's experience with tests of this nature that no increase in precision is obtained with the larger pipettes and the additional pipetting of buffer involved increases the time required to put in the test.

Procedure—The test-tubes are put in racks and covered with the inverted trays and, together with the pipettes, are dry-sterilized at 170° for $1\frac{1}{2}$ hours, cooled, and kept in reserve for use as needed.

The standard curve is established by pipetting the following amounts of standard solution (containing 50 Oxford units per ml) into a series of twelve test-tubes: 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0, 0, 0.08, 0.06, 0.04, 0.02 ml. These points correspond to values of 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, and 0 Oxford units.

In further series of tubes, volumes of the penicillin solutions to be tested are pipetted as follows: 0.16, 0.14, 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.08, 0.06, 0.04, 0.02 ml. As previously stated, the primary dilutions of the unknown solutions are made up so that their concentration falls in the range of 2 to 5 Oxford units per ml.

A flask containing sufficient basal medium to fill all the tubes in the test is selected and inoculated with the 14 to 18 hour inoculum. 65 ml of inoculum are used per liter of basal solution. Preliminary work indicates that within fairly broad limits the number of cells in the inoculum is not critical. However, further work is being done to determine whether a fixed number of cells per ml should be used.

A Fisher Volustat automatic pipetting machine is used to add the inoculated basal medium to the tubes. It is kept filled with 80 per cent alcohol when not in use and is rinsed out by pumping sterile water through it just before use. Both the suction and delivery tubes are put into the flask and the inoculated medium recirculated until all the bubbles are out of the delivery system and the medium is well mixed.

Two 3.5 ml shots of medium are then put in each tube in the test, the tubes covered with the inverted trays, and the racks put in a mechanically stirred water bath incubator at $37^{\circ} \pm 0.1^{\circ}$. The racks are left in the bath for an incubation time of 3 hours. Incubation times of $3\frac{1}{2}$ or 4 hours can be used, if times longer than 4 hours are employed the original inoculum must be cut down and more strict precautions for sterility observed.

The racks are removed at the end of the incubation time and steamed 10 minutes in an Arnold sterilizer or in an autoclave with the steam escape valve open. The purpose of this steaming is twofold: (1) it kills the cells and thus prevents additional growth from taking place during the reading

of the test and (2) the organisms are no longer dangerous to handle in the reading process. The racks are removed from the sterilizer and put in a water bath to cool. The tubes are shaken two at a time by covering the tops with the thumbs and giving them two sharp shakes to disperse the cells evenly.

Turbidimeter readings are made with a Lumetron model 402E equipped with a constant voltage transformer and with a special automatic emptying cell designed by the author.²

The broad band filter with a transmission peak at 5300 Å is being used at present but experiments with other filters are also being run.

Because of the high absorption of the medium used, a No. 7 reduction plate is used over the balancing photocell.

The reading of the tubes may be made in either of two ways on the Lumetron No. 402E colorimeter. We have set up the instrument so that a direct reading is taken from the galvanometer scale. When direct readings are used, a constant voltage transformer is absolutely essential, as the balancing photocell will not take care of all line fluctuations except when the galvanometer shows no current flowing in the balanced circuit. The exact details of operation will be supplied to anyone interested in using this particular instrument.

The second method of reading the tubes is to use the transmission dial to obtain the readings in the usual null point method. We do not use this method, because it is very much slower and does not improve the overall precision of the test.

The readings obtained are plotted against the number of Oxford units in the tubes and the points connected to form a smooth curve to be used as the standard (Fig. 1). The readings obtained on the unknown samples are compared against the standard curve and the number of Oxford units in the tube determined. (For examples of this calculation, see Table I.)

Results—Several methods of approach have been used to test the reliability of the method. These are (1) constancy of values obtained when potencies of samples are calculated from various portions of the standard curve (there should be no large drift in these values), (2) recovery experiments, (3) checking against the Oxford plate method which is apparently quite specific for penicillin, (4) tests of the reliability and reproducibility of single points in a replicate series, (5) reproducibility of assay figures on successive days.

* This cell consists of a specially selected Pyrex test-tube 18 mm. in diameter which has a 5 mm. tube fused to the bottom and connected by a rubber tube to a vacuum flask. A pinch clamp on the rubber tube is pressed to empty the tube after a sample has been read. This tube assembly is supported in a Saran tube containing windows $7/8 \times 11/32$ inch aligned in the light path of the instrument. The upper portions of the Saran tube and Pyrex tube stick up above a bakelite cover far enough so that it is unnecessary to cover the tube to prevent light from getting to the photocell.

TABLE I
Example of Test and Calculations

Standard curve (see Fig 1)		
Standard 5.0 Oxford units per ml	Oxford units per tube	Galvanometer reading
ml		
0.12	0.6	81
0.10	0.5	73.5
0.08	0.4	66.8
0.06	0.3	56
0.04	0.2	42.5
0.02	0.1	28
0	0	10
0	0	10.5
0.08	0.4	67
0.06	0.3	56.1
0.04	0.2	42
0.02	0.1	27.5

Commercial sample original solution contained 23.3 mg per liter solution diluted 1.3

Volume in tube (a)	Galvanometer reading (b)	Oxford units read from standard curve (c)	Oxford units per ml $\frac{(c)}{(a)}$	Average
ml				
0.16	58.5	0.320	2.00	
0.14	53.9	0.282	2.01	
0.12	48.1	0.240	2.00	
0.10	42	0.196	1.96	
0.08	36	0.157	1.96	
0.06	30.5	0.122	2.04	
0.04	24	0.082*	2.05	
0.02	14			
0.08	38	0.17	2.12	
0.06	31.9	0.13	2.16	
0.04	24	0.082*	2.05	2.04
0.02	15.1			

$$\frac{2.04 \times 3}{0.0233} = 262 \text{ Oxford units per mg}$$

* Ordinarily only points falling between 0.1 and 0.5 Oxford unit are used in calculations, as points outside this range give somewhat erratic results. In this case the points are included for the sake of illustration, since they show agreement with values obtained at other levels.

Data illustrating these points follow

Effect of Incubation Time—In general there seems to be little difference whether 3, 3½, or 4 hour incubation times are used (see Table II). Perhaps in testing for slight differences in potency the 3½ or 4 hour incubation may be preferable but for most purposes a 3 hour period is adequate. In a

few emergency cases incubation times as short as 2 hours and 10 minutes have been used to give approximate results

A comparison assay obtained on various samples at three incubation periods is given in Table II

TABLE II
Effect of Time of Incubation on Assay Values

Sample	Potency calculated		
	3 hrs incubation	3½ hrs incubation	4 hrs incubation
Commercial penicillin, Oxford units per mg	310	303	316
Diluted plant liquor, Oxford units per ml	3 64	3 60	3 63
Sample from flask fermentation, Oxford units per mg	189	191	196

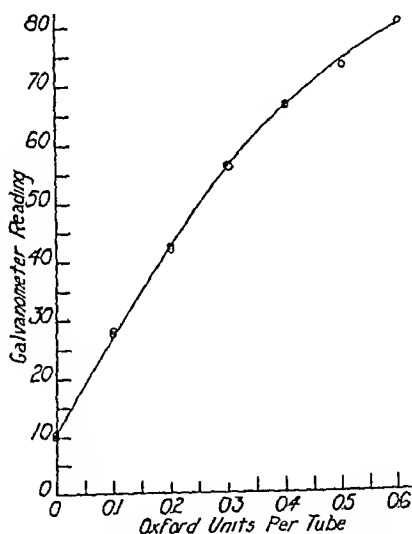


FIG 1 Typical standard curve for penicillin assay The galvanometer readings are arbitrary, i.e., they are not in per cent transmission

The example in Table I is an illustration of lack of "drift" of values over about a 4-fold range when a partially purified sample is used for assay. A similar test on a diluted plant liquor sample recently run in routine testing gave the results shown in Table III.

Recovery Experiments—Since most difficulty in obtaining specificity in microbiological materials is encountered with impure solutions, recovery tests were carried out on fermentation liquor

3 Hour Incubation—Solution A, diluted fermentation liquor, 3.06 Oxford units found per ml. Solution B, equal volumes of standard solution (5.0 Oxford units per ml) and Solution A were mixed and assayed. Theoretically this mixture should contain $(5.0 + 3.06)/2 = 4.03$ Oxford units per ml, actually found by assay, 4.06.

4 Hour Incubation—Solution A, fermentation liquor (as above), 3.15 Oxford units found per ml. Solution B, same as Solution B above. Theo-

TABLE III
Diluted Plant Liquor Sample

Volume ml	Oxford units	Oxford units per ml
0.14	0.525	3.75
0.12	0.45	3.75
0.10	0.388	3.88
0.08	0.33	4.10
0.06	0.232	3.87
0.04	0.147	3.68
0.02		
0.08	0.31	3.88
0.06	0.242	4.03
0.04	0.141	3.53
0.02		
Average		3.83

TABLE IV
Comparison with Oxford Plate Method and Series Dilution

The results are expressed in Oxford units per mg

Commercial penicillin sample	This method	Oxford	Series dilution
A	246	257	240
B	348	352	403
C	371	354	404
D	348	324	359
E	296	286	315

retically this mixture should contain $(5.0 + 3.15)/2 = 4.08$ Oxford units per ml, actually found by assay, 4.19.

Checks against Other Methods—Many samples of fermentation liquor have been run and these usually check within 2 to 10 per cent of the Oxford values. Several other samples are listed in Table IV for comparison. (The author wishes to acknowledge the kind cooperation of Dr. Ernest Weber of this laboratory who ran the Oxford tests for comparison.)

Reproducibility of Single Points—A standard curve was set up with six replicate points at each of the following points 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, and 0.0 Oxford units per tube. These were read in the usual manner and the mean and average deviation from the mean were calculated. The Oxford equivalent of the average deviation was calculated and from this the average percentage deviation of the single points was determined. While this number of points is not suitable for strict statistical analysis, the results obtained give a fairly good indication of the degree of reliability of single points. The percentage deviations obtained were as follows: 2.7, 1.9, 1.9, 6.3, 7.2, and 4.8 per cent at the 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1 Oxford unit levels respectively. Since an assay value is usually composed of six to eight points, the deviations tend to cancel and give an average value which is, of course, closer to the true value than that of single points.

Reproducibility from Day to Day—A comparison of reproducibility of this method and the Oxford and series dilution methods is shown in Table V.

TABLE V
Reproducibility of Assays from Day to Day

The results are expressed in Oxford units per mg

Method	1st day	2nd day	3rd day
Series dilution	399	348	338
Oxford	337	349	303
This method, 4 hrs incubation	316	307	299
" " 3½ " "	303		
" " 3 " "	310		

Effect of Inoculum—The amount of inoculum and its exact age are not critical within fairly wide limits. Three tests were run on a diluted plant liquor sample with inocula which had grown 6½, 24, and 72 hours at 37°. The assay values obtained were 4.06, 4.13, and 4.18 Oxford units per ml respectively. The Oxford test value was 4.16 on the same liquor.

Effect of Seitz Filtration—A solution of the standard was passed serially through five separate micro Seitz filters. Although there was a considerable reduction in volume due to absorption by the filter pads, there was no appreciable adsorption of the penicillin. The standard curve obtained from the filtered solution was almost exactly superimposable on the curve obtained from the standard solution which was only filtered once.

DISCUSSION

During the time this method was under investigation three other short period tests have appeared. In one of these (3) *Bacillus adhaerans* is employed

and has the advantage of being completely non-pathogenic. However, the test must be run in Erlenmeyer flasks which must be shaken throughout the incubation period. The agreement between the method and the Oxford plate method is not particularly good.

In another test (4) a strain of β -hemolytic streptococcus is employed which is not well suited to routine testing because of its high pathogenicity.

The third method (5), which is similar to that of Foster (1) and to that presented in this paper, for some reason does not achieve the reproducibility of points that a turbidimetric method is capable of giving. This may be seen in the non-superimposability of the standard curves. The use of optically matched tubes for growing the organisms is satisfactory for small numbers of tests but is not advantageous when many routine tests must be made per day.

Since the series dilution method and the Oxford plate method are both used at this laboratory, it has been possible to compare this short time turbidimetric method with these methods under actual operating conditions.

It has been our experience that the series dilution method requires the least amount of time and equipment. However, the results are not precise enough to yield accurate values unless duplicate tests of several different initial dilutions are run on each of 2 or 3 days. Since this involves twelve to eighteen tests, the initial advantage of the small amount of time required to put in the test is completely overcome. Single tests may be used when only a rough approximation of the penicillin content is required.

In using the modified Oxford plate method, Dr. Weber of this laboratory has found the precision of the test to be improved by the use of a daily standard instead of a "composite" standard often used. The cost of equipment for this test is very high because of the large number of Petri dishes and ground glass cylinders involved. The precision is in general quite good but occasionally results on one day or on certain samples may be quite far out of line. The exact cause of this is difficult to determine but research is being carried out to try to eliminate it.

The equipment cost of the short time turbidimetric method is less than that of the Oxford test if many samples are to be run, but if only a few samples are run the cost is greater owing to the cost of the colorimeter. The precision of the test is somewhat better than that of the Oxford method. The time required for putting in the test and reading it is approximately the same. The potency of the material does not have to be known as closely to put in the turbidimetric test as for the Oxford.

In developing the method presented in this paper, two factors were kept in mind, accuracy and speed (not only in cutting down the incubation time but also in the time required to put in the samples and read them).

To achieve accuracy, the amount of penicillin in the tubes must be the sole limiting factor in the growth of the organisms, i.e., all other variations should be eliminated. We have tried to obtain this by several means. (1) An automatic pipetting machine is used for putting medium into the tubes. This makes possible the filling of twenty-four tubes per minute, so that there is no large time gap between the filling of the tubes in the first and last parts of the test. (2) A mechanically stirred water bath incubator is used because all the tubes come to the same constant temperature rapidly and stay there for the incubation period. Owing to poor heat transfer, an incubator is not satisfactory. (3) A balanced cell photoelectric colorimeter equipped with a constant voltage transformer gives good stability in the reading process. (4) All cells are killed practically simultaneously in all the tubes by steaming.

Speed in the test is helped by the automatic pipette but the most crucial point is in the reading of samples. The cell used here empties by vacuum and 60 tubes may be read and recorded in 15 to 16 minutes. (This includes the time taken in rinsing the tube with clear medium between different samples.)

SUMMARY

A turbidimetric assay procedure for penicillin has been developed which is not only rapid but also more precise than the Oxford plate method. Several labor-saving devices have been incorporated to reduce the time necessary for putting in the test and reading it. The method has been checked quite thoroughly to establish its dependability.

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THE MICROBIOLOGICAL DETERMINATION OF FREE CHOLINE IN PLASMA AND URINE

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Many chemical methods for the quantitative estimation of choline have been described. The colorimetric modification of the reineckate method of Beattie (1) has proved successful only with relatively high concentrations of choline. The sensitivity of the reineckate method has been greatly increased by Marenzi and Cardini (2). With this method it is possible to determine choline in samples containing as little as 15 γ . However, in our laboratory this method was found to be unsatisfactory for the determination of free choline in body fluids. Available chemical methods are either very laborious or are suitable for the determination of only total choline in blood.

The need for a suitable method for the determination of free choline in body fluids and tissue extracts is stressed in a recent review by Best and Lucas (3). Such a method would certainly aid in studying the metabolism of this physiologically important substance.

Recently a microbiological method suitable for the quantitative estimation of small amounts of choline was described by Horowitz and Beadle (4). In this method the choline present in lecithin possessed approximately one-half of the activity of free choline. *dl*-Methionine also interfered with the assay when present in excess of 0.1 mg. per 25 ml. of the culture medium, however, this could be eliminated by adsorption on permutit. An adaptation of the microbiological method appeared to offer the best possibility of measuring the free choline in blood and urine.

Preliminary tests showed that most of the free choline in blood was present in the plasma and that the cells contained only a small proportion. For example, in a sample of sheep blood the plasma contained 36 γ of free choline per ml., while the cells contained only 5 γ per ml. For this reason the method described here is for plasma, however, there is no reason to believe that it would not be equally satisfactory when applied to laked blood.

Procedure

Blood Plasma—To 5 ml. of plasma in a 50 ml. centrifuge tube are added 20 ml. of acetone to precipitate the lecithin. The resulting precipitate is

removed by centrifugation, and the supernatant liquid decanted into a beaker. The acetone is evaporated off over a steam bath. The remaining liquid is made up to a convenient volume, usually 50 ml, with water. Aliquots of 1 and 2 ml, representing 0.1 and 0.2 ml of plasma, are added to the flasks containing the choline-free medium, described by Horowitz and Beadle (4). The inoculation with *choliness*, incubation, and measurement of growth response are carried out as described in the original method (4).

Urine—10 ml of undiluted urine are passed through a column containing 1 gm of permutit. This step is essential to remove interfering foreign growth stimulants. The adsorbed choline is then eluted with 10 ml of 5 per cent sodium chloride. In the case of human urine 1 and 2 ml of the eluate representing equivalent amounts of urine were found to contain adequate amounts of choline to give the desired growth response. Evidence will be presented later showing that all of the choline in urine is in the free form.

EXPERIMENTAL

In order to determine whether or not all the lecithin was precipitated by the acetone treatment, the total choline of the plasma was determined. Then the free choline of the plasma and the total choline of the acetone precipitate were determined. The results per ml of plasma were total choline 150 γ , free choline 40 γ , and total choline on the acetone precipitate 0.1 γ . All but 9 γ were accounted for in the free choline and the acetone precipitate. This is well within the range of accuracy claimed for the original method. Using larger amounts of acetone did not alter the results. The addition of acetone to the supernatant liquid prepared by mixing 5 ml of plasma and 20 ml of acetone did not give a precipitate, indicating that the 1:4 ratio of plasma to acetone is adequate to remove all of the lecithin.

Recovery Experiments—Known amounts of choline chloride were added to blood plasma and urine. These were then analyzed for choline by the method described. Both total and free choline were determined on the plasma. For the determination of total choline the plasma was first autoclaved with 3 per cent sulfuric acid for 2 hours at 15 pounds pressure, and the sulfuric acid removed with barium hydroxide. The urine samples were each from different individuals.

From the data in Table I it is apparent that the recovery of added choline came well within the range reported by Horowitz and Beadle. In the plasma the recovery was essentially as good whether on the basis of the free or total choline. The average recovery for the plasma for both the total and free choline was 100 per cent. The recovery from urine was slightly more variable than for plasma, but can be considered to be quite satisfactory.

Effect of Adsorption—*dl*-Methionine in high concentrations stimulates the growth of *cholincless*. Other substances may be present in plasma and urine which would interfere with the assay. Adsorption on permutit has been shown to be a satisfactory means of removing such substances (4). Tests were carried out to determine whether adsorption is a necessary step in the determination of free choline in plasma and urine. Assays were made on urine and acetone-treated plasma before and after adsorption on permutit. The results for plasma (Table II) are essentially the same irrespective of whether or not the acetone-treated plasma was passed through the adsorption column on permutit. The average free choline for the plasma from four different species was 34.7 γ per ml for both the adsorbed and non-adsorbed samples. It is evident from this that plasma does not contain

TABLE I
Recovery of Choline

Sample	Type of choline	Choline content of sample	Choline added	Total	Found	
		γ per ml	γ per ml	γ per ml	γ per ml	per cent
Plasma, horse	Free	40.0	10.0	50.0	51.0	102
" "	Total	150.0	10.0	160.0	161.0	101
" beef	Free	40.0	20.0	60.0	63.0	105
" "	Total	165.0	20.0	185.0	183.0	99
" sheep	Free	33.0	20.0	53.0	52.0	98
" "	Total	107.0	30.0	137.0	133.0	97
Urine, human	Free	7.0	5.0	12.0	11.4	95
" "	"	5.6	10.0	15.6	15.8	101
" "	"	8.0	15.0	23.0	21.0	91
" "	"	8.0	20.0	28.0	29.0	103

significant amounts of substances that interfere with the microbiological determination of choline.

Samples of urine from eight individuals were assayed for choline both before and after adsorption on permutit. In every case the choline values were slightly lower after adsorption. The average choline value per ml of urine before adsorption was 6.6 γ , while the value after adsorption was 6.0 γ . This difference is not large, but it was very consistent and cannot be considered to be due to experimental error. When substantially higher levels of choline occur in urine, adsorption may not be essential, but at the levels encountered here it definitely enhances the accuracy of the determination.

Effect of Acetone Treatment of Urine—Since lecithin is not considered to be a normal constituent of urine, it presumably would not be necessary to treat urine with acetone for the determination of free choline. The choline content of urine is so low that it does not permit autoclaving with

sulfuric acid as in the determination of total choline in plasma. In the method for total choline there is considerable dilution. This would result in such a low level of choline in the hydrolyzed urine that the growth response would not be measurable.

TABLE II
Effect of Adsorption on Free Choline Values

Sample		No adsorption	After adsorption
		γ per ml	γ per ml
Plasma	Horse	40	42
	Sheep	33	32
	Beef	40	38
	Pig	26	27
Average		34.7	34.7
Urine	Human	8.0	7.0
	"	7.0	6.2
	"	5.6	5.1
	"	6.0	5.6
	"	5.1	4.6
	"	6.5	5.7
	"	4.2	3.8
Average		6.6	6.0

TABLE III
Choline Content of Human Plasma and Urine

Subject	Plasma		Urinary excretion
	Free	Total	
	γ per ml	γ per ml	mg per 24 hrs
A	44	260	7.1
B	74	305	9.0
C	75	286	8.2
D	56	350	5.6

The possible presence of bound choline in the form of lecithin in urine could be tested by adding acetone to the urine. This was done as described for the determination of free choline in plasma. The average choline content of four different samples of urine, run in quadruplicate, before treatment with acetone was 6.8 γ per ml and 6.6 γ per ml after treatment with acetone. This affords confirmatory evidence that free choline only is present in urine.

Effect of Storage—In order to determine whether or not the choline content of plasma changes during storage several samples of plasma were stored at approximately 7° for 6 days. The results showed that there is no change in the free choline content of plasma during storage at the temperature indicated. The values on the stored samples were virtually identical with the values for the samples when fresh.

Choline Content of Human Blood and Urine—Blood was obtained from four adult healthy males and the urine from the same individuals was collected over a 24 hour period. In addition to the free choline content of the plasma, the total choline was determined so that the latter values obtained by the microbiological method could be compared with values available in the literature. The values for total choline in the four samples of human blood (Table III) ranged from 260 to 350 γ per ml of plasma. These values are of the same order as the single figure of 238 γ of choline per ml of human plasma obtained colorimetrically by Marenzi and Cardini (5). The free choline content of human plasma ranged from 44 to 75 γ per ml. These values are somewhat higher than those for other species (Table II).

The daily urinary excretion of choline for the four individuals ranged from 5.6 mg to 9.0 mg. These individuals were presumably receiving adequate diets, but they were not on the same dietary regimens. The relationship between choline intake and the daily renal excretion remains to be determined.

SUMMARY

The determination of free choline in plasma and urine by the microbiological method is described. The method appears to be equally applicable to the determination of total choline in blood.

Free choline in blood occurs chiefly in the plasma, with only a relatively small amount in the cells. The free choline in human plasma ranged from 44 to 75 γ per ml. This is somewhat higher than values observed in the horse, sheep, and cow.

The choline in urine exists entirely in the free state. The daily urinary excretion of choline of four adults ranged from 5.6 mg to 9.0 mg.

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THE DEPOSITION AND STORAGE OF α -TOCOPHEROL IN ABDOMINAL FATS*

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In a previous paper (1) it was reported that the rendered body fats of rats which had been raised from the time of weaning to the age of 100 days on a vitamin E-free diet were abnormally susceptible to oxidation at the C=C linkages by atmospheric oxygen. More specifically, oxygen absorption measurements at 100° were characterized by the almost complete absence of an induction period. It was further found that the addition of a single dose of 200 mg of α -tocopherol to the diet of rats on the vitamin E-free regimen restored the stability of their body fats to normal levels. Attempts to deposit various antioxidants other than the tocopherols in the adipose tissues of such rats via the diet have failed (1)¹. These and other observations indicated that tocopherols are the only antioxidants that occur normally in the adipose tissues of rats, and that they are derived exclusively from the diet.

At the present time a sensitive, specific, and widely applicable procedure for the chemical determination of tocopherols is lacking. The above conclusions immediately suggested an experimental technique for studying quantitatively the behavior of tocopherols in adipose tissues under various conditions, pending the development of a satisfactory chemical method. The present paper is concerned with the application of this technique and extends observations made previously.

EXPERIMENTAL

All of the rats used in the following experiments were females raised from the time of weaning on a vitamin E-free diet of the following percentage composition: sucrose 52.5, casein 19.1, lard 19.1, salts² 4.3, yeast 5.0, and approximately 500 i.u. of vitamin A concentrate to each rat twice monthly. The lard used in composing the diet had been rancidified by blowing air through it at 75–100° until it had attained a peroxide content

* Aided by grants from the Hormel Research Foundation, the National Dairy Council, and the Graduate School of the University of Minnesota.

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² Salt mixture described by McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 33, 63 (1918).

of 20 or more milliequivalents per kilo, thus insuring the destruction of tocopherols occurring naturally in the lard

The general procedures followed in sacrificing the rats and in rendering the fats were identical with those previously described (1) Only the abdominal fat depots exclusive of the mesentery were used, reference will hereinafter be made to them simply as "abdominal fats"

The oxygen absorption measurements were made with a partly insulated standard Warburg respirometer at 100°, essentially in accordance with the technique described by Johnston and Frey (2) Butyl phthalate was used as the manometer fluid The flasks were shaken through an amplitude of 3 cm at the rate of 100 to 110 oscillations per minute Readings were made at 10 minute intervals In the beginning of a run the flasks were thoroughly flushed with oxygen from a cylinder to remove air and then equilibrated in the bath for 10 minutes at atmospheric pressure before the capillaries were closed Under the conditions used in these measurements the induction period of a fat is almost completely independent of the oxygen pressure, slight variations from day to day in the atmospheric pressure at which the flasks were equilibrated were therefore disregarded

In obtaining the measurements recorded in Figs 1 and 2, ordinary conical Warburg flasks of 15 to 18 ml capacity were used These flasks were later replaced by a more satisfactory cylindrical type (2)

Iodine numbers were determined in duplicate on many of the fats, with the mercuric acetate modification of the Wijs method

Reference Curve 1—The abdominal fats from a group of the vitamin E-deficient rats were pooled and rendered Oxygen absorption measurements revealed that the fat had a negligible induction period and its tocopherol content was therefore assumed to be nil Synthetic α -tocopherol (Merek) was dissolved in absolute alcohol and aliquots were added to weighed amounts of the fat to give solutions ranging in concentration from 0 to 80 γ of tocopherol per gm of fat After complete removal of the alcohol by evacuation at approximately 75°, replicate oxygen absorption measurements were made on 0.2 ml samples

With the exception of the fat containing no added tocopherol, it was found in all cases that there was a period of low and relatively uniform oxygen uptake which ended in a sudden and rapidly accelerated increase in the rate The rise was sufficiently sharp to define the induction period within the time interval between readings, i.e., 10 minutes The behavior of many animal fats in this respect is in marked contrast to that of various tocopherol-containing vegetable oils and appears to have been satisfactorily explained by Golumbic (3)

The observed induction periods are recorded in Fig 1 as a function of the tocopherol concentration in micrograms per gm of fat In common with

the observations made by numerous investigators on the antioxygenic behavior of α -tocopherol (and other phenolic antioxidants), it is found that a given increment of the antioxidant is less effective in lengthening the induction period as the antioxidant concentration increases

The fat used in this experiment is ideally adapted as a substrate for use in studying some of the properties of fat antioxidants, unlike other natural or processed fats, it appears to contain inappreciable amounts of either antioxidants or prooxidants

Deposition of α -Tocopherol in Abdominal Fat Tissues—Vitamin E-deficient rats whose history was identical with that of the group used in obtaining the reference curve were fed single 50 mg doses of α -tocopherol. The tocopherol was dissolved in the ethyl esters of corn oil fatty acids

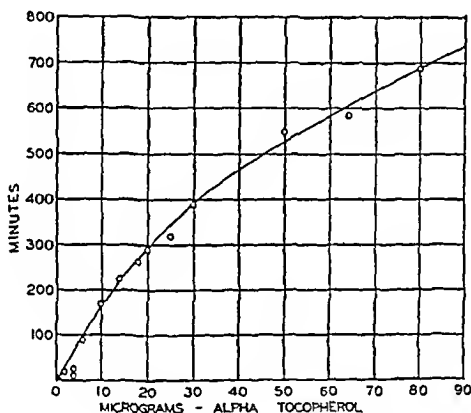


FIG 1 Reference Curve 1 Relation of induction period to the concentration of α tocopherol

from which all unsaponifiable material had been removed, in a concentration of 50 mg per ml, and 1 ml of the solution was fed to each rat by stomach tube. The rats were then sacrificed at various intervals in groups of two, and the fats were removed and rendered as soon as possible. In Fig 2 the induction periods are recorded as a function of the interval between the administration of tocopherol and sacrifice. Each point on the curve represents an average of at least two oxygen absorption measurements. The irregularities in the distribution of the points are largely due to variations in the small groups of rats.

The iodine numbers of the fats in this series were within the limits 70.1 and 70.8, except the 63 day sample which had an iodine number of 71.5. It appears justifiable to assume that the differences in induction periods are almost entirely attributable to differences in tocopherol content and

only negligibly related to differences in composition. On the basis of this assumption, the induction periods have been converted to tocopherol concentrations by means of the reference curve in Fig 1, and the results are given in Fig 3.

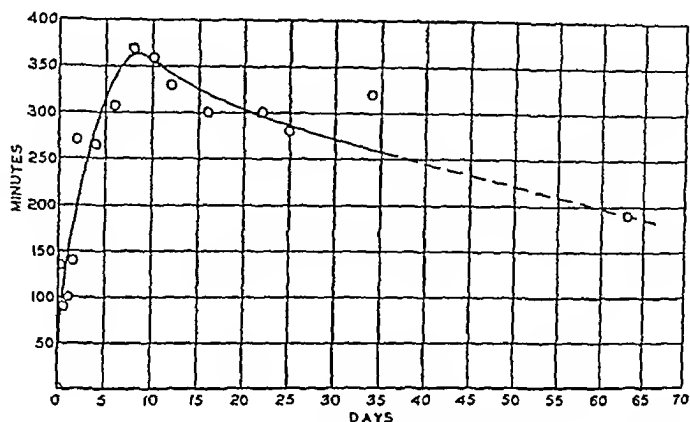


FIG 2 Relation of induction period to the time interval between the feeding of α -tocopherol and sacrifice of the animal

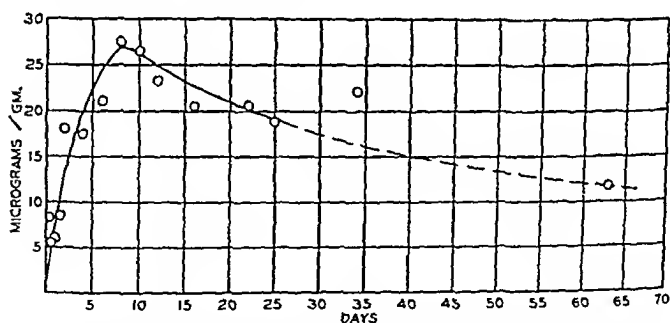


FIG 3 Relation of the concentration of deposited α -tocopherol to the time interval between the feeding of α -tocopherol and sacrifice of the animal

Miller and Burr (4) have shown that α -eleostearic acid of tung oil attains a maximum deposition in the body fats of rats approximately 48 hours after feeding 0.6 to 0.9 gm of tung oil. Since the maximum deposition of α -tocopherol is not attained until 7 to 10 days after oral administration (Fig 3), it seems clear that a temporary deposition or storage of tocopherol occurs elsewhere in the animal, followed by a subsequent gradual redistribution.

Of the 50 mg that were fed, the maximum concentration found in the rendered fat was approximately 27 γ per gm. Although the tocopherol is distributed in many of the organs and tissues, the body fats are normally one of the sites of relatively high concentration (5), and no greatly predominant site of storage has yet been discovered. Some evidence has been presented (6) showing that when excessive amounts of tocopherols are fed large fractions are excreted in the feces. Therefore, as will be discussed in further detail later, at least some of the fat depots must be regarded as possible important sites of tocopherol storage, in spite of the low concentrations deposited relative to the amounts fed.

Reference Curve 2—On the basis of results previously published it had not been anticipated that large amounts of α -tocopherol could be deposited in

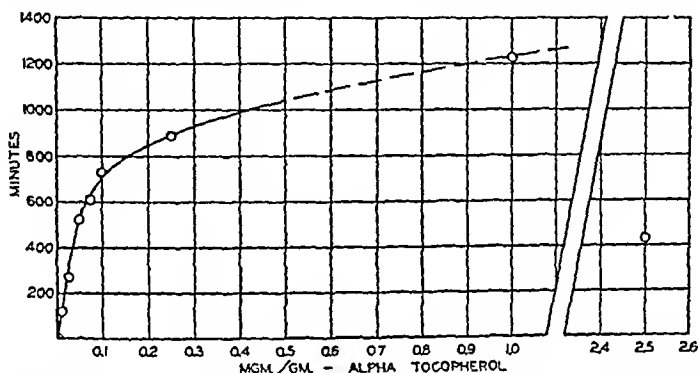


FIG 4 Reference Curve 2 Relation of induction period to the concentration of α -tocopherol

the adipose tissues by feeding. For the next experiment, when larger doses of tocopherol were fed, it became desirable to extend the reference curve given in Fig 1. Another group of vitamin E-deficient rats was therefore sacrificed and a new reference curve prepared that included much higher concentrations of α -tocopherol. Triplicate oxygen absorption determinations were made on 0.4 ml samples with the 70 ml cylindrical flasks. The results are given in Fig 4.

The induction periods were sharply defined at all tocopherol concentrations, even at 2500 γ per gm. However, at the higher concentrations the rate of oxygen absorption during the induction period was greater. This is in harmony with the observations made by Swift, Rose, and Jamieson (7), who found that the rate of peroxide formation in methyl oleate, methyl linoleate, and methyl esters of cottonseed oil was greater during the induction period at higher tocopherol concentrations.

Golumbic (3) reported that above 2.0-10 per cent concentration (1000 γ per gm) in cottonseed oil esters and in lard the further addition of tocopherols shortened the induction period. Fig 4 demonstrates that a maximum in the induction period is similarly found in the abdominal fats from rats used in these experiments at approximately the same level of α -tocopherol concentration.

Effects of Feeding Large Amounts of α -Tocopherol—Four groups of rats, two in a group, with the same history as those used in preparing reference Curve 2, were fed daily doses of 50 mg of α -tocopherol until total intakes of 50, 125, 250, and 500 mg had been provided. The groups were sacrificed 7 days after the last feeding. The induction periods of the rendered

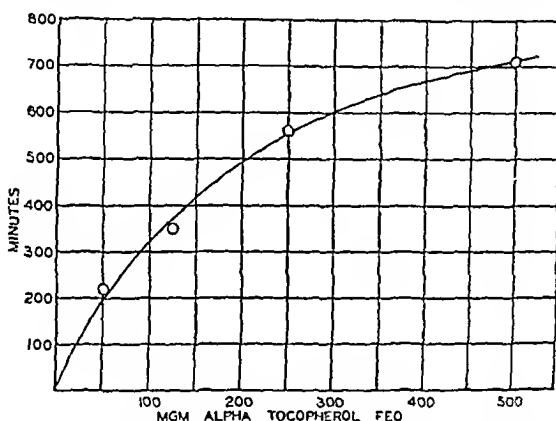


FIG 5 Relation of induction period to the amount of α -tocopherol fed

fats are given in Fig 5. As before, the variations in iodine numbers were negligible.

The induction periods in relation to the amounts of tocopherol fed tend to level off at the higher intakes. However, when the induction periods are converted to equivalent tocopherol concentrations (Fig 6), it is seen that the tocopherol concentration is still increasing rapidly at the largest quantity fed. Moreover, the rats were all killed 7 days after the last feeding on the assumption that the maximum concentration had been attained. In spite of the fact that no more than 50 mg were administered daily, it is conceivable that at the larger total intakes the maximum concentration of approximately 97 γ per gm recorded in Fig 6 at an intake of 500 mg must be regarded as a low value.

Attempts were made to measure the amount of tocopherol deposited when very small amounts were fed. The results were inconsistent, partly

because an insufficient number of rats was used to average the relatively greater individual variations encountered at the low levels. However, the administration of 1 mg of α -tocopherol³ markedly influenced the stability of the fat, producing an induction period of 43 minutes in the fat from two rats. The administration of 10 mg produced an induction period of 113 minutes in the fat from two rats.

The conversion of induction periods to tocopherol concentrations by means of the reference curves involves the important assumption that the α tocopherol is deposited in the adipose tissues in a form that has the same antioxidant activity as α -tocopherol dissolved directly in rendered fat.

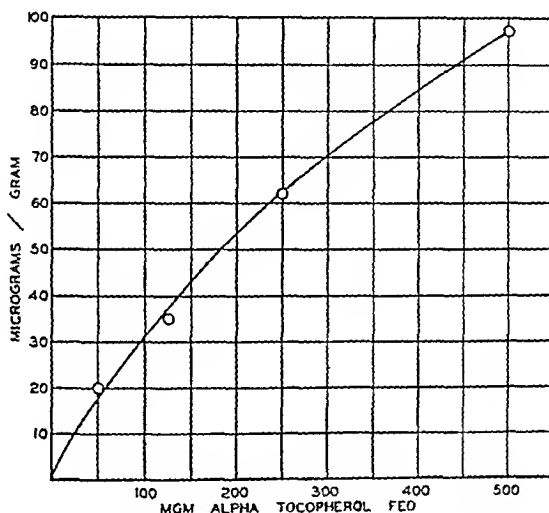


Fig 6 Relation of the concentration of deposited α -tocopherol to the amount fed

In order to test the validity of this assumption, the fats from the rats described above which had been fed 0, 250, and 500 mg of α -tocopherol were analyzed by a chemical method. The chemical method used, as yet in the process of development,⁴ is a modification of the Furter-Meyer method (8). By the method here being described the three fats were found to contain 0, 62, and 97 γ of α -tocopherol per gm, respectively, the chemical method gave corresponding figures of <3, 52, and 98. It seems highly probable, therefore, that α -tocopherol is deposited largely in unaltered form in adipose tissues, or, if not, then in a form which has substantially the same antioxidant activity.

³ The amount needed to cure sterility in the majority of young female rats

⁴ Chipault, J. C., Lundberg, W. O., and Burr, G. O., in preparation

DISCUSSION

The increasing deposition of α -tocopherol obtained by feeding increasingly large doses appears to contradict the results previously reported (1) in which it was found that the addition of 0.25 ml of 15 per cent mixed tocopherols twice weekly for 1 month to the diet of rats reared on a normal diet did not appreciably increase the stability of the abdominal fats. The earlier experiment differed from the present one in several important respects: (a) The additional supplement of tocopherols was in the form of a concentrate of mixed tocopherols of unknown composition, (b) the animals had been fed on normal diets from the time of weaning, and (c) the induction periods were measured by other methods. As will be indicated in the following discussion, the first of these considerations appears to be particularly important.

Mason (5), using a bioassay method, studied the relative vitamin E contents of various organs and tissues of rats reared on low vitamin E, high vitamin E, and excess vitamin E diets. In the latter two diets the vitamin E was added in the form of raw wheat germ and mixed tocopherols, respectively. It was estimated that the three diets supplied 4, 100, and 10,000 times the minimum daily requirements. Comparing the high vitamin E with the low vitamin E diet, he found that the former caused a 14-fold increment in liver storage, but increased the vitamin E content of other tissues, including body fats and muscle tissues, by only 3 to 4½ times. The relative differences in these ratios appeared to be even greater in a comparison of rats fed excess vitamin E with those fed the low vitamin E diet. (As is the case with our own present results, the ratios observed by Mason for body fats apparently disagree with the results of our earlier experiment cited above.) He concluded that the metabolic needs of other tissues are satisfied at the expense of liver storage and that the liver constitutes the chief repository for vitamin E when the intake is optimal or greater.

Hines and Mattill (6), using a chemical method, reported that in rats which had been fed vitamin E concentrates (mixed tocopherols) equivalent to 100 mg of tocopherol daily for an unspecified period, the average tocopherol content of the livers was 42.3 γ per gm, and of muscle tissue, 11.9. The diet presumably contained considerably more tocopherol than the high vitamin E diet used by Mason. In rats on normal diets the figures were 22.1 and 7.5, respectively, and in rats on vitamin E-deficient diets, 2.6 and 4.8. The tocopherol content of the vitamin E-deficient diet used by these investigators was presumably as low or lower than that of the low vitamin E diet used by Mason. Contrary to the latter's findings, the ratio of the liver content between the rats on high vitamin E and the vitamin E-deficient rats is lower than the ratio of the muscle content. Considering

the very great difference in the vitamin E content of the high vitamin E and the vitamin E-deficient diets, the remarkably low ratios calculated from the results of Hines and Mattill are comparable to the results we obtained in abdominal fats in our earlier experiment, but appear to disagree with the results of the present experiment. These investigators conclude also that hepatic storage may explain the protracted vitamin E deprivation that rats may undergo without demonstrating the symptoms of vitamin E deficiency, probably because the *absolute* differences in liver contents are greater than those in muscle.

Having discussed apparent contradictions between the results of the above investigators, our previous results, and the results of the present investigation, we find it necessary to emphasize that the contradictions may be more apparent than real, since there were major differences in the experimental conditions and techniques used. The purpose of the discussion has been to illustrate that the functions of deposition and storage cannot be ascribed primarily to any one organ or tissue on the basis of results heretofore obtained. Before a clear understanding of the phenomena of deposition and storage of tocopherols can be achieved it appears likely that it will be necessary (a) to study the contents of all tissues at various definitely known intakes of tocopherol and (b) to study the pure tocopherols individually. It may be found that the relationships in deposition and storage between various tissues will vary with both of these factors.

In the present investigation, in which synthetic α -tocopherol alone was used as the source of vitamin E, it was found that an intake of 500 mg resulted in a minimum deposition of 97 γ per gm of rendered abdominal fat. In the absence of knowledge concerning the content of other tissues in this experiment, no specific conclusions can be drawn. Since Hines and Mattill found, however, that an administration of vitamin E concentrate equivalent to 100 mg daily resulted in only 42.3 γ per gm in the liver, the abdominal fats as well as the liver must be regarded as possible important storage sites under certain conditions.

The precise function or functions of tocopherols in animal tissues are still obscure. The interruption of pregnancy and damage to the sexual organs produced by Kudryashov and coworkers (9-11) and others (12) by feeding or injecting decomposition products of rancid fats suggest that tocopherols may prevent an *in vivo* oxidation of fats similar to *in vitro* rancidification (with or without enzymes). Other investigators (13) dispute their results, ascribing the effects to conditions associated with the meager stores of vitamin E in the animals used. Houchin and Mattill (14) and Houchin (15) postulate that α -tocopherol, in the form of a phosphate, plays an important rôle in a complicated enzyme system in muscle tissue.

SUMMARY

A technique has been described by means of which changes in the tocopherol content of the adipose tissues of rats may be quantitatively studied.

A maximum deposition of α -tocopherol in the abdominal fats of vitamin E-free rats, exclusive of the mesentery, is not achieved until 7 to 10 days after feeding a single 50 mg dose. The concentration of tocopherol decreases slowly thereafter, reaching a level of about one-half of the maximum after 2 months.

The feeding of α -tocopherol in various amounts up to 500 mg results in the deposition of increasing amounts in the abdominal fats. The amounts deposited indicate that the fat depots may be major sites of storage under certain conditions.

Apparent discrepancies between the results of various investigators indicate that further work must be done in which various known amounts of a single tocopherol are fed before any final conclusions may be drawn concerning the relationships between various tissues in the deposition and storage of tocopherol in the rat.

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SOLUBILITY OF ADULT AND FETAL CARBONYLHEMOGLOBIN OF THE COW

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It is well known that the hemoglobins obtained from members of different species are different, and that the differences originate in the globin portion of the molecules. It is not entirely clear, however, whether the hemoglobins obtained from different members of the same species are the same, or whether the blood of a given individual contains more than one kind of hemoglobin molecule, and, if so, whether there are changes in the amounts of the different kinds with the age and condition of the individual.

Various experiments have been reported which deal with these questions. In 1927 Valer (1) made analytical measurements of the percentage of iron and sulfur in purified hemoglobin from different sources. In all cases the amount of iron was the same (0.33 per cent), but the amount of sulfur varied from one species to another. In the cat and cow, samples from different individuals were always alike, but in the horse and dog the sulfur content varied from one sample to another in a way which was interpreted to mean that dog hemoglobin is a mixture of several different kinds of molecules and that different individuals differ in respect to their hemoglobin. These results received confirmation in later studies of Kaiser (2), Timar (3), and Simonovits and Balassa (4). Schenck (5), in 1930, reported differences in the amino acid content of hemoglobin from different human individuals but it is questionable whether the differences do not lie within the limits of error of his analyses. Schenck has also reported a difference in the rate of digestion by pepsin of adult and fetal hemoglobin of man. On the basis of studies of the rate of denaturation of hemoglobin by strong alkali, von Kruger (6) and subsequently von Kruger and Bischoff (7) and Bischoff and Schulte (8) concluded that fetal hemoglobin in man is different from that of the adult, being more resistant to denaturation. Similar conclusions have been reached by Haurowitz (9, 10) and by Brinkman and his associates (11), who state that the blood of new born infants contains, in addition to the fetal type of hemoglobin, 20 per cent of a less resistant form which they identify with that of the adult. Brinkman and his associates also state that adult human blood contains 8 to 20 per cent of a resistant hemoglobin which may or may not be identical with fetal hemoglobin. These conclusions are supported by later studies of Brinkman and Jonxis

(12) on the rate of spreading of hemoglobin in monolayers. In these studies of Brinkman it was found that the resistant form of hemoglobin in fetal blood disappears 7 months after birth and is replaced by the resistant form in adult blood at $2\frac{3}{4}$ years.

There are numerous other studies directed to the problem of establishing a difference between adult and embryonic hemoglobins. Pernier and Jannelli (13) made optical measurements of crystals of hemoglobin from adult and new born human beings. They reported that the crystals from adults were biaxial, those from the new-born, uniaxial. These results were later confirmed by Haurowitz (10). On the other hand, careful spectroscopic studies of Jongbloed (14) in the ultraviolet region showed no difference between hemoglobin from adults and new-born. The greatest amount of work, however, has to do with the oxygen equilibrium. Haselhorst and Stromberger (15), in connection with the problem of oxygen secretion by the placenta, made measurements of the oxygen dissociation curves of maternal and fetal whole blood of man. They determined the oxygen affinity¹ of the two kinds of blood in relation to pH and showed that at a given pH the affinity was higher in the case of fetal blood than in the adult. They believed that the difference is the result of an adaptation on the part of maternal blood, resulting in a decrease of oxygen affinity. In 1933, McCarthy (16) made gasometric measurements of the oxygen dissociation curves of purified goat hemoglobin dissolved in M/15 phosphate at pH 6.8. All samples of maternal hemoglobin gave the same curve, with $n = 2.2 \pm 0.3$ ². In fetal hemoglobin the value of n was found to be 2.0 ± 0.4 and the oxygen affinity was approximately twice that of maternal hemoglobin. The two kinds of hemoglobin gave rise to different membrane potentials, but the osmotic pressure measurements showed no evidence of a difference of molecular weight. In a very recent communication (17) McCarthy reported similar results on solutions of fetal and adult hemoglobin of the sheep. The hemoglobin of the adult has a lower oxygen affinity and a higher value of n than fetal hemoglobin, but there is no difference between the osmotic properties of the two. In 1934 Barcroft (18) and others made an extensive study of fetal and maternal whole blood from goats. In agreement with the work of McCarthy, they found that the oxygen affinity of maternal blood was less than that of the embryo. The difference was attributed in part to a difference of pH and in part to a

¹ The term oxygen affinity used in this discussion is the reciprocal of the value of the oxygen pressure corresponding to 50 per cent saturation of the protein with oxygen.

² This is the n which occurs in Hill's equation describing the combination of hemoglobin with oxygen. The equation is $y/100 = kp^n/(1 + kp^n)$ where y denotes percent age saturation of hemoglobin with oxygen, k and n are constants, and p is the partial pressure of oxygen.

real difference in the hemoglobin molecules. In the same year, Hall (19), using a spectroscopic method, obtained oxygen dissociation curves of solutions of goat hemoglobin, maternal and fetal, at a concentration one-one-hundredth that in blood. At pH 6.8 the oxygen affinity of fetal hemoglobin was seven-fourths that of maternal blood. Similar results were obtained with hemoglobin of incubating chicks (20), for which it was shown that there was a gradual decrease in oxygen affinity during development. In 1935 Haurowitz (10) showed that with human hemoglobin in dilute solution (0.15 per cent) the curve obtained for the adult lies to the left of that for the embryo, but that the relationship is reversed when the hemoglobin is present in corpuscles. In 1936 Hill and Wolvekamp (21) showed that the dilution of adult human hemoglobin might lead to a 2-fold increase in oxygen affinity, without any considerable change in the shape of the curve. Fetal hemoglobin was found to be much less affected by dilution. Similar effects were observed with other animals. This effect of dilution on oxygen affinity has been quite recently confirmed by Ginezensky (22). Still more recently, McCarthy (23) has shown by gasometric measurements that the affinity of embryonic human hemoglobin in concentrated solution is slightly less than that of maternal hemoglobin, but that when the hemoglobin is in the corpuscles the relationship is reversed.

In view of this rather complicated picture and of the importance of the question of the existence of different kinds of hemoglobin, we have undertaken a study of the solubility of adult and embryonic cow hemoglobin. Solubility represents perhaps the most significant criterion of chemical identity, and is a property which can be studied fairly easily. The present paper presents results which, though somewhat incomplete, are believed to be decisive.

Cow hemoglobin was employed in all the experiments. The crystallizations of the protein and the subsequent equilibrations were all made in strong phosphate solutions of $\text{pH} \cong 6.8$, in the salting-out region of the solubility curve. Approximately 1000 cc of blood from adults and 500 cc of fetal blood were used for each experiment. This was collected from the slaughter-house in bottles containing 4 per cent trisodium citrate in amounts equal to approximately 10 per cent of the volume of blood. In addition to citrate a very small amount of toluene was added to minimize bacterial growth. The blood was cooled as soon as possible after it was received, and the cold blood was then saturated with carbon monoxide. After this the cells were washed three or four times with cold 1.85 per cent sodium chloride, being centrifuged each time in the cold. The washed cells were then laked by being shaken after addition of approximately one-fifth of their volume of toluene and one-fifth of their volume of water. After the laked cells were centrifuged to remove the stroma, the supernatant

liquor was syphoned off and filtered in a Buchner funnel through No 5 filter paper covered with Celite, or sometimes simply through paper. The clear filtrate was then saturated with carbon monoxide and stored in the cold as a stock solution.

When an aliquot of cold stock solution was added to concentrated ice-cold phosphate buffer, pH 6.8, the amounts being such as to give a final concentration of phosphate equal to about 2.15 moles per liter, an amorphous precipitate resulted at once, which, on standing and being shaken, became crystalline. Sometimes, after the precipitate had stood overnight in the cold, crystals could be seen attached to the side of the flask, which were an appreciable fraction of a mm in length. It was apparent that the amount of precipitate obtained with solutions prepared from blood of adults was always greater than that obtained with solutions from embryonic blood and that the crystals from the latter were more soluble than those from the adult.

We give the following description of an orienting experiment which illustrates this. Two stock solutions of adult and embryonic hemoglobin were prepared by the method described above. A phosphate buffer was made up from K_2HPO_4 and KH_2PO_4 , containing 4.346 moles per liter. The ratio K_2HPO_4/KH_2PO_4 was 0.577/0.423 and the density at 28° was 1.458. The calculated pH was very close to 6.8. This buffer was saturated with carbon monoxide. To each of two 100 cc volumetric flasks, labeled Flasks A and E, for adult and embryonic hemoglobin respectively, were added exactly 50 cc of this buffer. The flasks were then chilled to -4° and diluted to the mark with the cold stock hemoglobin solutions. An amorphous precipitate at once developed, which was much greater in Flask A than in Flask E. Both flasks were left in the cold overnight. On the next morning some crystals were apparent in each flask, but the bulk of the material appeared to be amorphous. After the flasks were shaken and allowed to stand for an hour or two, the precipitate in each had become completely crystalline so far as could be observed. When samples from Flasks A and E were centrifuged for half an hour at 3000 R P M, the crystals, being lighter than the mother liquor, collected at the top. The clear liquid underlying the crystals from the blood of the adult was colorless, that underlying the crystals from embryonic blood was a deep pink. After these liquids were syphoned off, the crystals were washed three times with a solvent made by diluting the stock phosphate buffer with water to twice its volume. The molar concentration of this solvent in total phosphate was 2.173 and the density 1.245 at 25°. During the second washing the crystals were left in contact with the solvent overnight. Throughout the process it was evident after each centrifugation that the clear liquid underlying the crystals of embryonic carbonylhemoglobin was always much

darker in color than that underlying the crystals of carbonylhemoglobin from the adult, and that the former crystals disappeared much faster than the latter. At the end of the third washing the crystals of embryonic carbonylhemoglobin were nearly gone.

In view of these results, further and more careful experiments were done in which the concentrations of the solutions were determined spectrophotometrically. For this purpose either of two instruments was employed. One of these was the spectrophotometer made by the Central Scientific Company but modified by the substitution of a much more sensitive photoelectric measuring device. In the measurements made with this instrument the nominal slit width was 37 Å. The other was the Beckman photoelectric quartz spectrophotometer. In the measurements made with this instrument the nominal slit width was 8 Å. All the measurements were made at a wave-length of 5000 Å and concentrations were calculated by taking the value of the absorption coefficient of cow carbonylhemoglobin as 5.50×10^4 sq cm per equivalent, where the weight of the equivalent is 16,700 (24). This means that if the depth of the absorption cell is 1 cm, as in all our measurements, the concentration of hemoglobin in gm per 100 cc may be obtained by multiplying $\log I_0/I$ by 0.303. It may be noted that at a wave-length of 5000 Å the absorption coefficients of oxy- and carbonylhemoglobin are almost identical.

The first experiment was carried out as follows. Two stock solutions, Solutions A and E, containing adult and fetal hemoglobin respectively, were prepared in the usual way except that, before filtration through Celite, the solutions obtained from the laked cells were dialyzed against water to remove traces of salt. After saturation with carbon monoxide, the concentrations of hemoglobin in these two stock solutions were measured with the spectrophotometer. That in Solution A was 22.0 gm per 100 cc, and that in Solution E was 19.1 gm per 100 cc. The density of Solution A was 1.0493, which, on the basis of a partial specific volume of hemoglobin of 0.749, would mean a concentration of 20.6 gm per 100 cc. The concentrations of these two stock solutions were equalized by diluting Solution A with water to $22.0/19.1 = 1.15$ times the original volume. After this the concentrations of the two solutions were checked with the Beckman instrument and found to be, Solution A, 17.05 gm per 100 cc, Solution E, 17.10 gm per 100 cc. These two figures may be taken as equal within the limits of experimental error. The difference between the concentrations of Solution E as measured with the two photometers must be ascribed to factors involving the instruments themselves, but it is not relevant to the present experiments to inquire into it further. A phosphate buffer like that described in the preliminary experiment was made up and saturated with carbon monoxide. Exactly 50 cc of this were added to each of two

425 cc centrifuge cups These were then cooled in an ice-salt mixture and to one were added exactly 50 cc of the cold stock Solution A To the other, an equal amount of cold Solution E was added The cups were then stoppered, put in the ice chest, and left overnight On the next morning they were placed in a rotator in a water bath at 7° and left rotating 4 days At the end of this time the cups were centrifuged and the supernatant liquids were syphoned off and filtered in the cold The concentrations of hemoglobin in the supernatant liquids were then determined with the spectrophotometer and found to be as follows Solution A, 0.228 gm per 100 cc, Solution E, 2.59 gm per 100 cc While these equilibrations were being carried out, two additional precipitations were made in which 18 cc of adult or embryonic hemoglobin were added to 20 cc of phosphate buffer in smaller centrifuge cups The solutions were then equilibrated by rotating the cups in the 7° bath for 3 days, and were removed and measured at the same time as the solutions in the large cups The concentrations of hemoglobin in this second pair of solutions were as follows Solution A, 0.0636 gm per 100 cc, Solution E, 0.616 gm per 100 cc

These results reveal the considerable difference in solubility between adult and fetal carboxyhemoglobin However, it should be pointed out that the nature of the experiment accentuates this difference, as the protein precipitates, taking with it a certain amount of water of crystallization, it gives rise to an increase in the phosphate concentration in solution which tends to lower the solubility of the protein further The effect is greater in the case of the less soluble protein

In a second experiment the same stock solutions, Solutions A and E, and the same phosphate buffer were employed to make up a set of solutions in test-tubes To each tube was added a certain volume of phosphate buffer, a certain volume of stock hemoglobin solution, Solution A or E, and a certain volume of water, the water, like the phosphate buffer, having been saturated with carbon monoxide The sum of the volumes of water and hemoglobin solution was in each case equal to the volume of the phosphate buffer These solutions were then equilibrated by rotating the test-tubes for several days in the bath at 7° At the end of this time, the contents of all the tubes were filtered to remove crystals and the concentrations of the hemoglobin in the filtrates were measured with the Beckman spectrophotometer In each case the measured concentration of hemoglobin was tabulated against the hypothetical concentration calculated from the way in which the corresponding test-tube was made up, on the assumption that all the protein remained in solution In these calculations the concentration of hemoglobin in both stock solutions was taken as 17.07 gm per 100 cc, the value obtained with the Beckman instrument, since the concentrations of dissolved hemoglobin were measured with this in-

strument No account was taken of the slight volume contraction resulting from mixing the liquids, since this is of the order of only 1 to 2 per cent and is *very* nearly constant for all the tubes Its only effect would be to alter slightly the slope of the theoretical straight line shown in Fig 1 This experiment was carried out twice, although lack of stock Solution E limited the number of points obtained the second time The results are shown graphically in Fig 1

This type of solubility experiment is different from that used by Northrop So long as the solution is unsaturated, the concentration of dissolved protein remains equal to the calculated concentration of added protein, subject only to the slight effect of volume changes discussed above As soon as the saturation point is reached and crystals begin to form, the volume of the liquid phase is reduced, and, owing to the water of crystallization

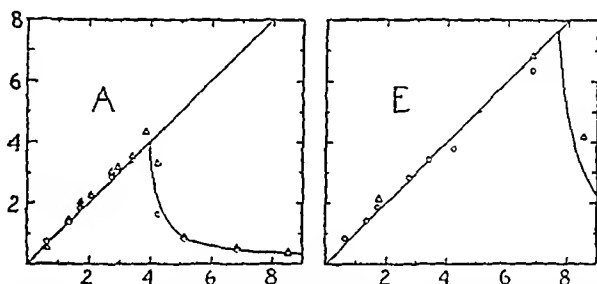


Fig 1 Solubility of carbonylhemoglobin from the adult cow (Curve A) and embryo (Curve E) The ordinates give the concentration of hemoglobin in solution in gm per 100 cc, the abscissae give the total amount of hemoglobin present in gm per 100 cc O, first run, Δ , second run

of the hemoglobin, the proportion of phosphate to water in this phase is increased This leads to the phenomenon shown in Fig 1, involving a sharp drop in the concentration of dissolved protein as more stock solution is added The effect provides a sharp "end-point" corresponding to saturation There is also another factor in the situation to be considered As more stock solution and correspondingly less water are added, the ratio of total water in the system to total phosphate decreases whether or not there is any precipitate Thus at the point of saturation the solvent is more concentrated in phosphate in the case of the more soluble protein

This experiment makes it possible to calculate with fair accuracy the solubility of each of the two hemoglobins in a solvent of known ionic strength, different for each It may be estimated from Fig 1 that saturation occurs at a concentration of 4 gm of protein in 100 cc in the case of the hemoglobin of the adult and of 7.7 gm of protein in 100 cc in the case of

embryonic hemoglobin These concentrations would result from combining the following quantities of water, stock hemoglobin solution, and phosphate buffer

	Adult cc	Fetus cc
Stock solution	23 4	45 1
Water	26 6	4 9
Phosphate buffer	50	50

From these figures, taking account of densities, we calculate the following quantities of materials to be present in the two systems

	Adult	Fetus
Hemoglobin, gm	4	7 7
Water, gm	85 65	83 10
Phosphate, mole	0 2173	0 2173

These figures mean that, at 7°, 4 gm of the hemoglobin of the adult will dissolve in a solvent consisting of 85 65 gm of water and 0 2173 mole of phosphate in which K_2HPO_4 KH_2PO_4 = 577 423, and that at the same temperature 7 7 gm of fetal hemoglobin will dissolve in a solvent consisting of 83 10 gm of water and the same amount of phosphate It is a simple matter to calculate the ionic strengths of these two solvents All that is needed for this is a knowledge of the densities These were estimated on the basis of density measurements on other phosphate solutions of known composition as 1 257 and 1 265 These two figures give for the ionic strengths of the two solvents the following values carbonylhemoglobin solution for the adult, 4 90, for the embryo, 5 05

A final experiment remains to be considered Stock Solutions A and E of adult and embryonic hemoglobin were prepared as already described, without the step involving dialysis The same phosphate buffer was employed as before, and this was saturated as before with carbon monoxide Stock Solutions A and E were added cold to equal volumes of phosphate buffer After the precipitations were complete, samples of the mother liquor were withdrawn and centrifuged The densities were then measured and found to be, Solution A, 1 2926, Solution E, 1 2806 A phosphate buffer was prepared by diluting the original buffer so that the concentration was 2 57 moles per liter, the ionic strength 5 54, and the density 1 2880 This was used as a solvent to wash the two sets of crystals Six washings were carried out with this solvent over a period of 10 days at 7° During each washing the crystals were equilibrated with the solvent by rotating the container in the constant temperature bath At the end of each equilibration the material was centrifuged, the solvent syphoned off, put aside for measurement, and replaced with fresh solvent At the end of the process the last four washings were filtered, and, after being suitably diluted,

measured with the Beckman spectrophotometer The results are given in Table I

These experiments show that embryonic cow hemoglobin is more soluble than adult and would seem to furnish decisive proof that the two proteins are distinct It is possible to make use of the collected data to obtain crude salting-out curves for the two hemoglobins by plotting \log_{10} solubility against ionic strength For this purpose we assemble the following

TABLE I
Hemoglobin Concentration As Measured with Beckman Spectrophotometer

Washing No	Adult	Embryo
	gm per 100 cc	gm per 100 cc
3	0 012	0 080
4	0 006	0 062
5	0 016	0 070
6	0 012	0 076
Average	0 0115	0 072

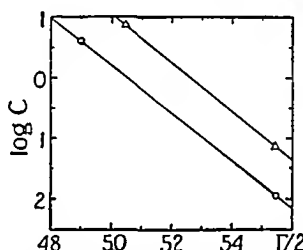


FIG 2 Solubility of the carbonylhemoglobin from the adult cow \circ and fetus \triangle in relation to the ionic strength, $\Gamma/2$ The ordinates give the log concentration in gm per 100 cc

data for the solubility, S , in gm per 100 cc, of the two kinds of hemoglobin in relation to Γ , the ionic strength of the solvent per liter

Adult hemoglobin, $\Gamma = 4.90$, $S = 4.0$,³ $\Gamma = 5.54$, $S = 0.0115$
 Fetal " " = 5.045, " = 7.7, " = 5.54, " = 0.072

The result of the plot is shown in Fig 2 The curves may be interpreted in terms of the equation

$$\log S = \beta - K' \frac{\Gamma}{2}$$

³ It should be mentioned that a preliminary and somewhat less reliable experiment on the solubility of the hemoglobin of the adult at an ionic strength of 4.68 would indicate a considerably lower solubility than corresponds to that in Fig 2

in which β and K' are constants, by assuming the following values of the constants adult hemoglobin, $\beta = 20\,237$, $K' = 4$, fetal hemoglobin, $\beta = 21\,033$, $K' = 4$. The values of β would indicate that, in the range of ionic strengths studied, the solubility of fetal hemoglobin is about 6.3 times that of the adult. However, since the curves drawn in Fig. 2 are each based on only two points, too much weight cannot be attached to the exact values of the constants, although there would seem to be no question of the greater solubility of fetal hemoglobin. Whether or not the adult and fetal hemoglobin preparations both represent a single kind of molecule or a mixture of two or more different kinds is a question which cannot be settled on the basis of these experiments. In the latter alternative it would seem likely that the crystals are solid solutions.

SUMMARY

Solubility measurements have been made on carbonylhemoglobin of adult and fetal cow blood. In strong phosphate buffers, of ionic strength 4.9 to 5.5 and pH 6.8, the hemoglobin from embryonic blood is more than 6 times as soluble as that from the adult. This would indicate that the two hemoglobins are distinct.

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WATER-SOLUBLE VITAMINS IN SWEAT*

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Claims have been made that significant amounts of the water-soluble vitamins may be lost in sweat. In view of the importance of this problem in tropical and desert nutrition and the conflicting reports (4, 9, 12, 20), we have made an extended investigation, using a variety of refined techniques for collecting and stabilizing sweat and for estimating vitamins. In addition to ascorbic acid, dehydroascorbic acid, thiamine, riboflavin, and nicotinic acid, which have been studied by one or more other workers, we have estimated diphosphothiamine and the fluorescent pigments of Najar and Holt and collaborators (15), F_1 and F_2 . A complete summary of the literature in this field will be found at the end of the present paper.

EXPERIMENTAL

Production of Sweat—In each experiment healthy young men living on a good normal diet sweated approximately 1 liter per hour while marching in a heated room according to a régime commonly employed in this laboratory (18). Desert conditions were simulated at 43° and 30 per cent relative humidity, moist tropical at 35° and 85 per cent relative humidity. Eleven men in all stages of acclimatization (ranging from none at all to complete) were used as subjects.

Collection of Sweat—Sweat was collected in two ways. First, elbow length rubber gloves were worn for half an hour. Second, sweat was scraped off the body with a glass vessel. Precautions and special points of technique necessary in the case of individual substances are discussed below.

Methods and Results

Estimations were made on fresh sweat of the following substances: ascorbic acid, dehydroascorbic acid, thiamine, diphosphothiamine, riboflavin, nicotinic acid, F_1 , and F_2 . The results are summarized in Table I, which shows negligible amounts of all. No differences were detected between sweat from unacclimatized and acclimatized men.

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Ascorbic and Dehydroascorbic Acids—The results for ascorbic and dehydroascorbic acids in sweat are shown in Table II, which demonstrates the

TABLE I
Summary of Estimations of Vitamins in Sweat

Substance	No of samples	Concentration in sweat	
		Average	Range
		mg per 100 ml	mg per 100 ml
Ascorbic acid	31	0 00	0
Dehydroascorbic acid	16	0 02	0-0 2
Thiamine and diphosphothiamine	15	0 00	0
Riboflavin	10	0 00	0
Nicotinic acid	40	0 05	0-0 1
Fluorescent pigments F ₁ and F ₂ of Najjar and Holt and collaborators (16)	5	0 00	0

TABLE II
Ascorbic and Dehydroascorbic Acids in Sweat

The results are expressed in mg per 100 ml

Subjects	No of experiments	Ascorbic acid		Dehydroascorbic acid	
		Indophenol	Methylene blue	Indophenol	Methylene blue
F S	2	0		0 1-0 4	
J P	5	0	0	0 -0 5	0 -0 2
J S	5	0	0	0 -0 4	0
M C	3	0	0	0	0
G P	3	0	0	0	0
R J	3	0			
F C	3	0			
M P	2	0			
H B	2	0			
G T	2	0			
M M	1	0			
Total of experiments	31	31	16	18	16
Highest		0	0	0 5	0 2
Lowest		0	0	0	0
Average		0	0	0 2	0 02

absence of ascorbic acid and the presence of dehydroascorbic acid in small amounts

Ascorbic acid was estimated by the method of Mindlin and Butler (13) with use of 2,6-dichlorophenol indophenol and the Evelyn photoelectric

colorimeter and also by the method of Butler and Cushman (3) by means of the reversible decolorization of methylene blue by strong light in the presence of ascorbic acid. Dehydroascorbic acid was converted by the hydrogen sulfide reduction of Bessey (2) into ascorbic acid which was then estimated by one or both of the above methods. In the estimation of dehydroascorbic acid, the methylene blue method is better than the indophenol method, because it admits of accurate correction for the effect of

TABLE III

Behavior of Ascorbic and Dehydroascorbic Acids in Sweat

In each typical experiment ascorbic acid was added to the vehicle at 0 time. AA represents ascorbic acid, DAA dehydroascorbic acid. The results are given in mg per cent.

Experiment*	Time of exposure	Temperature of exposure	Vehicle						
			Water + 6 per cent HPO ₃		Fresh sweat		Sweat + 6 per cent HPO ₃		Boiled sweat
			AA	DAA	AA	DAA	AA	DAA	AA
	min	C							
A	0	38	3 0		3 0		3 0		3 0
	5	38			2 2				2 3
	15	38			1 7				0 8
	30	38	3 0		0 8		3 0		0 0
"	0	5	2 1		2 1		2 1		2 1
	30	5			1 0				1 1
	60	5	2 0		0 8		2 1		0 7
	120	5	2 0		0 8				0 3
B	0	39	3 1	0	3 1	0	3 1	0	
	1	39			0 8	0 8			
	10	39			0 8	0 7			
	20	39			0 1	0 7			
	30	39	3 0	0	0 0	0 6	3 1	0	
	45	39			0 0	0 4			
	60	39	3 1	0	0 0	0 2	3 1	0	

* Experiment A illustrates the effects of temperature, stabilizers, and boiling of sweat on the destruction of added ascorbic acid. Experiment B illustrates the relation between ascorbic and dehydroascorbic acids after addition of ascorbic acid.

traces of hydrogen sulfide. For this reason we consider the methylene blue values the more representative of the true dehydroascorbic acid content of sweat.

The detection of ascorbic acid in sweat presents difficulties because of its rapid destruction. These difficulties are illustrated in Table III. Six points should be noted. First, ascorbic acid added to sweat disappears rapidly and at different rates in different samples. Second, if ascorbic acid

is added after sweat has been boiled for 5 minutes and then cooled to 38° or to 5°, the rate of destruction is greater than in fresh sweat at the same temperatures. Hence, enzymatic action is excluded. It is possible either that the ions of heavy metals are set free by boiling and are the destructive agents, or that traces of protein which delay the breakdown of ascorbic acid in fresh sweat are denatured by boiling. Third, cooling retards but does not entirely prevent the destruction of ascorbic acid. Fourth, addition of metaphosphoric acid to sweat stabilizes added ascorbic acid completely for at least an hour, even at 40°. Fifth, destruction of added ascorbic acid is accompanied by production of dehydroascorbic acid, but the sum of these two acids never equals the initial content of ascorbic acid. Sixth, destruction of dehydroascorbic acid proceeds at a relatively slow rate even in fresh sweat.

The above demonstration of the extremely rapid destruction of ascorbic acid in sweat at body temperature, with the simultaneous production of dehydroascorbic acid, was corroborated by pouring solutions of ascorbic acid down the arms of sweating subjects. In a typical experiment, an aqueous solution containing a known concentration of ascorbic acid was divided into three portions of 50 ml each. One portion was stabilized at once with 50 ml of 6 per cent metaphosphoric acid and was stored on ice. Another portion was kept in the hot room and was added to 50 ml of 6 per cent metaphosphoric acid at a rate approximating that at which the third portion was poured slowly and continuously down the arm of a sweating man, draining into 50 ml of 6 per cent metaphosphoric acid. The rate of pouring was 10 ml per minute. The arm was washed twice at the end with distilled water. Ascorbic and dehydroascorbic acids were then estimated in the iced control, in the hot room control, and in the solution that had been poured down the arm. When an absolute amount of 0.90 mg of ascorbic acid was poured down the arm, 0.67 mg was recovered as ascorbic acid and 0.23 mg as dehydroascorbic acid. When an absolute amount of 0.22 mg of ascorbic acid was used, 0.04 mg was recovered as ascorbic acid and 0.12 mg as dehydroascorbic acid. Three conclusions may be drawn from this type of experiment. First, ascorbic acid breaks down rapidly on the surface of a sweating arm, with concomitant production of dehydroascorbic acid. Second, the lower the concentration of added ascorbic acid, the more complete is the destruction of ascorbic acid. Third, even with low initial concentrations of ascorbic acid, the terminal value of dehydroascorbic acid is a reasonably satisfactory measure of the initial ascorbic acid.

In view of the instability of ascorbic acid added to sweat and because of the demonstrated stabilizing effect of metaphosphoric acid, certain precautions were taken in the collection of sweat. When gloves were used, 6 per cent metaphosphoric acid was placed in them before collection started.

When a glass vessel was used for scraping the body, the sweat was transferred as rapidly as possible to a cold brown bottle containing 6 per cent metaphosphoric acid. In control experiments both procedures allowed satisfactory recovery of ascorbic acid added in known amounts either to the glove or to the scraping vessel.

As Table II shows, no ascorbic acid was detected in thirty-one different samples of sweat, even when all precautions were observed. Under the same conditions dehydroascorbic acid was sometimes detected in small amounts. If significant amounts of ascorbic acid are lost in sweat, the vitamin must be destroyed before reaching the surface. Under these conditions we would expect relatively large amounts of dehydroascorbic acid to appear in the sweat, as is demonstrated in the experiments described above. However, only small amounts of dehydroascorbic acid actually do appear in sweat. Therefore, we conclude, first, that insignificant amounts of ascorbic acid are destroyed in the sweat ducts, and second, that even in a hard day's work, involving the loss of 10 or more liters of sweat, there is no significant loss of vitamin C by this route.

Thiamine and Diphosphothiamine—Thiamine was estimated by the method of Egaña and Meiklejohn (6), diphosphothiamine by an unpublished fluorometric method of Egaña and Robinson. Sweat was collected by all of the methods described above. In control experiments when thiamine was added to sweat in amounts approximating 10 γ per 100 ml, recovery was quantitative under the following conditions: (1) when added to sweat at body temperature, (2) when added to sweat containing 6 per cent of metaphosphoric acid, (3) when added to a glove worn for $\frac{1}{2}$ hour by a sweating man, and (4) when poured over the arm of a sweating man. It is clear, therefore, that thiamine, in contrast to ascorbic acid, is destroyed very slowly when added to sweat. Hence, the estimation of thiamine in sweat presents few difficulties.

Of fifteen samples of fresh sweat, ten assayed zero and in the rest neither thiamine nor diphosphothiamine was present in amounts exceeding the limits of sensitivity of the method, which is about 0.5 γ per 100 ml.

Riboflavin—Riboflavin was estimated by the method of Najjar (14). Sweat was collected by all of the methods described above with the following additional precautions against light: the heated room was lighted only by a 60 watt, orange, photographic dark room lantern and all vessels were of amber glass. In control experiments when riboflavin was added to sweat in amounts approximating 10 γ per 100 ml, recovery was 100 per cent in the incubation, glove, and arm experiments. In none of ten samples of sweat could any riboflavin be detected.

Nicotinic Acid—Nicotinic acid was estimated by the methods of Harris and Raymond (8) and of Kodicek (11). In our hands, nicotinamide could

be recovered quantitatively when added to water, but when added to sweat yields were erratic. It appeared that some interfering substances, varying in amount from sample to sample, rendered the assays somewhat unreliable. When sweat was concentrated *in vacuo* (see below) recoveries were more satisfactory, for unexplained reasons.

In forty fresh samples of sweat an apparent value of between 0.0 and 0.1 mg. of nicotinic acid per 100 ml. was detected, the average being 0.05.

Fluorescent Pigments of Najjar and Holt, F₁ and F₂ These pigments were estimated by the method of Najjar and Wood (16) with one modification. Adsorption was effected by shaking in a glass-stoppered mixing cylinder with 5 gm. of activated permutit instead of passing the sweat through an adsorption column. In none of the samples did any F₁ or F₂ appear.

Concentrated Sweat—Because analyses of fresh sweat indicated negligible quantities of vitamins, sweat was concentrated in an attempt to obtain a definitive estimate of the actual content of riboflavin, thiamine, and nicotinic acid.

TABLE IV

Stability of Vitamins Added to Sweat during Concentration by Vacuum Distillation

Portion	Recovery of added vitamins per cent of initial value		
	Thiamine	Riboflavin	Nicotinamide
Fresh, unconcentrated	100	100	100
Unconcentrated, heated 5 hrs. at 40°	100	90	100
Concentrated <i>in vacuo</i>	80	75	110

nicotinic acid. Fresh sweat was acidified with glacial acetic acid to 3 per cent. Vacuum distillation in the dark at 15 mm. of Hg and 40° concentrated this sweat 20 to 40 times in 5 hours. In order to determine the stability of the vitamins under these conditions, known amounts of thiamine, riboflavin, and nicotinamide were mixed with acidified fresh sweat, and the mixture was divided into three portions. One portion was analyzed at once. A second was heated at 40° in the dark for 5 hours. The third was concentrated 20 to 40 times, as described above. Table IV demonstrates that loss of thiamine and riboflavin was about 20 per cent, but nicotinamide was completely stable. Therefore, analysis of concentrated sweat should reveal essentially the true content of these vitamins. Analyses of five such samples gave the following average results: thiamine 0, riboflavin 0, and nicotinic acid 0.08 mg. per 100 ml., calculated in terms of fresh unconcentrated sweat.

Animal Assay for Thiamine in Concentrated Sweat—6 liters of fresh sweat were concentrated as described above and frozen at -6.6°. Dr. F. J. Stare

of the Department of Medical Nutrition, Harvard Medical School, kindly assayed this concentrate by its effect on the survival of rats deficient in thiamine. He reported that with two levels of intake there was no thiamine detectable by this method. On the average death was earlier among the rats receiving the higher dosage of sweat.

DISCUSSION

Consideration of all of the results reported since 1936 is assisted by reference to Table V, which shows how extremely various the estimates have been. In general, the early reports give higher values than the later, and three of the four most recent papers report negligible amounts of all the vitamins assayed. Some of the high results can probably be attributed to one or both of two factors: first, the analytical methods were not specific for the substances to be estimated, second, the sweat was not collected by techniques that insured against contamination.

Studies on sweat have an important bearing on nutritional requirements in hot climates in that they can elucidate one of the possible routes of vitamin depletion. The general conclusion to be drawn from the work of investigators who have used specific analytical methods as well as unexceptionable technique in collecting sweat is that loss in the sweat is not a significant factor in depleting the body's stores of water-soluble vitamins. Even under severe conditions that necessitate a daily loss of 10 to 15 liters of sweat, excretion of vitamins is considerably greater in the urine than in the sweat. Few observers have been seriously concerned over urinary loss as a cause of vitamin depletion, and we consider that they should be even less exercised over losses in the sweat.

SUMMARY

1 Water-soluble vitamins in sweat have been investigated by a variety of analytical methods and with a variety of methods for collecting and handling sweat.

2 The following substances could not be detected by chemical methods in any sample of fresh sweat: free ascorbic acid, thiamine, diphosphothiamine, riboflavin, and the fluorescent pigments F_1 and F_2 of Najjar and Holt.

3 Dehydroascorbic acid appeared in half of sixteen samples in amounts not exceeding 0.2 mg per 100 ml.

4 Nicotinic acid, as estimated by the method in which cyanogen bromide and an aromatic amine are employed, appeared in a majority of forty samples in a concentration not exceeding 0.1 mg per 100 ml.

5 Rat assay failed to show thiamine in samples of sweat concentrated *in vacuo* by a method proved to stabilize the vitamin reasonably well.

6 From a review of the work of those investigators who have employed

The results are expressed in mg or in micrograms per 100 ml

	Cornbleet <i>et al</i> , 1936 (5)	Bernstein, 1937 (1)	Zselyonka and Nánásy Mécay 1937 (22)	Wright and MacLenathem 1939 (21)	
Method of sweat induction	Thermal	Exercise	Thermal	Thermal	T
Method of sweat collection	Subject en- cased in rubber bag	Not described	Scraping into glass vessel with acid	Dripped into cold acetic acid	C

Ascorb					
Analytical method	Indophenol titration	Not measured	Indophenol ti- tration	Indophenol titration	In
Range, mg	0.55-0.64		0.1-0.2	0.024-0.186	

Dehydroas					
Analytical method	Not measured	Not measured	Not measured	Not measured	A
Range, mg					

Total ascorbic acid (ascor)					
Analytical method	Not measured	Indophenol ti- tration fol- lowing H ₂ S reduction	Not measured	Not measured	A
Range, mg		0.5-1.1			

	Hardt and Stull 1941 (7)	Slater 1942 (19)	Tennant and
Method of sweat induction	Exercise	Thermal	Thermal, t
Method of sweat collection	Not fully described	Scraped into glass vessel with acid	Sponge eq cold solu

Thia			
Analytical method	Thiochrome, fluoro metric	Thiochrome, fluoro metric	Thiochrome metric
Range, γ	8.2-150	0.1-0.7*	0.0

Ribo			
Analytical method	Not measured	Not measured	Microbiolo
Range, γ			

Pantethe			
Analytical method	Not measured	Not measured	Microbiolo
Range, γ			2

Nicotin			
Analytical method	Not measured	Not measured	Not measur
Range, mg			

* These observers also measured diphosphothiamine and found none

† These observers

Peck <i>et al</i> 1939 (17)	Hardt and Still 1941 (7)	Tennent and Silber 1943 (20)	Mickelsen and Keys 1943 (12)	Sargent <i>et al</i> cf Johnson 1943 (9)
hermal otton pledgets, no stabilizer	Exercise Not fully de scribed	Thermal, exer cise Sponge squeezed into cold H ₂ SO ₄	Exercise Rubber glove drained con tinuously into cold acetic acid	Exercise Rubber glove containing 6% HPO ₃ , scraped into cold HPO ₃
ic acid				
dophenol ti- tration, 2 samples	Indophenol photometric and titration	Indophenol ti tration	Indophenol photometric	Indophenol, pho tometric meth ylene blue, pho tometric
0.0862, 0.42	0.18-0.20	0	0-0.18	0
corbic acid				
ot measured	Not measured	Aniline, furfural	Not measured	Methylene blue photometric fol lowing H ₂ S re duction
		0-0.2		0-0.2
bic and dehydroascorbic)				
ot measured	Not measured	Aniline, furfural	Not measured	Methylene blue, photometric following H ₂ S reduction
		0-0.2		0-0.2
Silber 1943 (20)	Cornbleet <i>et al</i> 1943 (4)	Mickelsen and Keys 1943 (12)	Sargent <i>et al</i> cf Johnson 1943 (9)	
ercise squeezed into ene	Thermal Subject encased in rub ber bag, sweat con centrated	Exercise Rubber glove drained continuously into cold stabilizer, sweat con centrated	Exercise 1 Rubber glove con taining 6% HPO ₃ 2 Scraped into 6% HPO ₃ , sweat concen trated	
mine				
c, fluoro	Colorimetric, diazo tized ethyl <i>p</i> amino benzoate (cf (10)) <6-60	Thiochrome, fluoro metric	1 Thiochrome, fluoro metric 2 Rat assay 0*	
3-0.7		<0.2		
flavin				
gical	Microbiological	1 Microbiological 2 Fluorometric	Fluorometric	
1-7	3-30	<0.5	0	
mic acid				
gical -30	Microbiological 12-80	Not measured	Not measured	
ic acid				
ed	Microbiological 0.01-0.046	1 Microbiological 2 Chemical "Averaged ca 0.1"	C ₃ anogen bromide, aromatic amine 0-0.1†	

also measured F₁ and F₂ of Najjar and Holt and collaborators (15, 16) and found none

the most specific methods as well as an acceptable technique in collecting and handling sweat, it is concluded that loss in the sweat is not a significant factor in depleting the body's stores of water-soluble vitamins. Even under severe conditions, loss in the sweat is much less than excretion in the urine.

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OXIDATION OF FRUCTOSE BY BRAIN IN VITRO

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Fructose does not maintain the electrical activity of the cerebral cortex in the hepatectomized animal (1). However, whole brain (2) and cortex (3) oxidize fructose *in vitro*. These facts may be explained by assuming that the brain cells are impermeable to fructose *in vivo*, or that the oxidation of fructose is not concerned in the maintenance of cortical activity. The latter supposition makes it necessary to assume that the metabolism of fructose by brain differs from the metabolism of glucose, since glucose does support cortical activity in the hepatectomized animal (1).

In the present work the oxidation of fructose by brain preparations was studied *in vitro*. The data obtained indicate that the oxidation of fructose by brain *in vitro* follows the same pattern as the oxidation of glucose. Thus, the data support the hypothesis that brain cells are impermeable to fructose *in vivo*.

Materials and Methods

The brain was prepared as follows. A cat was killed by a blow on the head and decapitated. The brain was removed, homogenized with 30 ml of 0.04 M potassium phosphate, pH 8.0, containing 0.02 M magnesium chloride, and then squeezed through muslin. The pH of the mixture was about 7.0. 1 ml aliquots of this preparation were mixed with 0.8 ml of a solution containing the following substances in the concentrations indicated: 0.2 M nicotinamide, 0.004 M fumarate, 0.003 M adenosine 5-triphosphate or adenosine-5-phosphate, 0.09 M fluoride, 0.003 M diphosphopyridine nucleotide, 0.002 M thiamine pyrophosphate, and 0.05 M potassium phosphate, pH 8.0. The pH of the final mixture was about 7.2. The mixtures were incubated at 37° in air until the oxygen uptake had decreased to about 1 micromole per 10 minutes. Then 0.2 ml of water or substrate solution was added to each mixture, and the incubation continued in air or nitrogen. For experiments involving anaerobic production of lactate alone the homogenate was diluted five times with the phosphate-magnesium mixture, and then treated as indicated above, except with omission of fluoride and the preliminary incubation in air.

The oxygen uptake and carbon dioxide production were followed manometrically. After these measurements the mixtures were treated with 1 ml

of 2 N trichloroacetic acid and 7 ml of water, and then filtered. The filtrates were analyzed for lactate (4) substances binding bisulfite, i.e. carbonyl groups (5), inorganic phosphate (6) before and after hydrolysis in N acid at 100° for 10 and 180 minutes, inorganic phosphate formed by treatment with N alkali at room temperature for 20 minutes (7), and for total phosphate. Suitable controls were analyzed similarly.

The rate of formation of inorganic phosphate by acid hydrolysis furnishes presumptive identification of organic phosphates. The rates of hydrolysis of a number of organic phosphates and the inferences deducible from the rates have been summarized (8). In the present study hydrolysis during 10 to 180 minutes of a considerable portion of the organic phosphate obtained in a given experiment has been considered indicative of the presence of hexose phosphate and resistance to hydrolysis during 180 minutes indicative of phosphoglycerate.

Further identification of organic phosphate was made in some experiments as follows. 10-fold quantities of brain preparation and other material were treated as indicated above except that the gas exchange was not measured and the proteins were precipitated with 1 ml of 10 N trichloroacetic acid. A suitable aliquot of filtrate was analyzed for organic phosphate. After the addition of 0.1 volume each of ethyl alcohol and 1 M barium acetate the remainder of the filtrate was made alkaline to phenolphthalein with solid barium hydroxide. The mixture was centrifuged. The precipitate was suspended in 2 ml of water and acidified to Congo red paper with 10 N hydrochloric acid. The mixture was again made alkaline with barium hydroxide. The precipitate was collected by centrifugation, washed thrice with 1 ml portions of 0.1 M barium acetate, and then dissolved in dilute acid. The solution was assayed for fructose (9, 10), the amount and rate of hydrolysis of the organic phosphate, and for substances reducing Benedict's qualitative alkaline copper reagent. With this procedure about 80 per cent of the organic phosphate formed in a given experiment could be isolated as a barium salt.

Results

The aerobic oxidation of fructose by the brain preparation is illustrated by the data in Table I, Experiment 1, A. In this experiment oxygen and inorganic phosphate disappeared. The disappearance of inorganic phosphate signifies the formation of organic phosphate. The resistance to acid hydrolysis of the major fraction of the organic phosphate suggests that the product formed was phosphoglycerate. In a large scale experiment the organic phosphate isolated as the barium salt was not appreciably hydrolyzed by hot N acid in 180 minutes, did not contain fructose, and did not reduce the alkaline copper reagent. This likewise suggests that the product

of oxidation of fructose under the conditions employed was phosphoglycerate

Lactate was formed in the experiment given in Table I, but not in all such experiments. Incomplete inhibition of phosphoglycerate breakdown by fluoride might lead to the production of pyruvate, which might account for formation of lactate by the reaction indicated in Equation 3. The carbonyl compounds in the control at the beginning and the end of the

TABLE I

Oxidation and Phosphorylation of Fructose by Cat Brain in Vitro

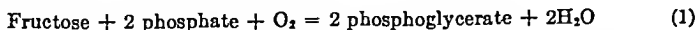
The preparations of brain used are described in the text. Fluoride was present in Experiments 1 and 2, but not in Experiment 3. The periods of incubation after addition of substrates were 30, 45, and 60 minutes for Experiments 1, 2, and 3, respectively. The data given are the differences between the observed and appropriate control values. The minus sign indicates disappearance of the substance in question, the plus sign indicates formation.

The results are expressed in micromoles

Conditions	Experiment No	Substrate	Oxygen	Carbon dioxide	Inorganic phosphate	Organic phosphate			Lactate	Carbonyl compound
						Hydrolyzed by v acid at 100 during 10-180 min	Not hydrolyzed by N acid at 100 in 180 min	Hydrolyzed by N alkali at room temperature in 20 min		
Aerobic	1, A	Fructose, 40	-2.5	0.0	-19.7	+1.3	+17.3	+5.7	+4.4	+3.4
	1, B	Pyruvate, 16.8	-5.5	+9.7	-2.1	0.0	+2.8	0.0	+2.7	-6.7
	1, C	Fructose, 40, + pyruvate, 16.8	-5.5	+8.8	-42.8	+13.5	+15.1	+2.3	+10.9	-8.7
Anaerobic	2, A	Fructose, 40, + pyruvate, 18.5	0.0	0.0	-10.1	+1.0	+8.0	0.0	+9.6	-8.0
	2, B	Fructose, 40	0.0	0.0	-1.1	+0.5	0.0	0.0	0.0	0.0
	2, C	Pyruvate, 18.5	0.0	0.0	0.0	0.0	0.0	0.0	+2.4	-3.7
	3	Fructose, 40	0.0	0.0	-8.0	+5.5	+1.5	0.0	+10.0	0.0

experiment were not present in sufficient amount to account for the formation of lactate.

An equation that may account in part for the results is



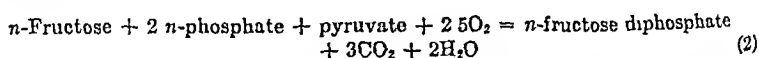
This reaction implies the direct oxidation of triose phosphate which has been demonstrated with brain preparations (11, 12). The formation of

triose phosphate in the present experiment is indicated by the production of carbonyl compounds and organic phosphate hydrolyzed by alkali (7) Phosphorylation coupled with oxidation of triose phosphate has been observed in other tissues (13, 14) In brain (15) and other tissue (7) triose phosphate is formed from fructose diphosphate Therefore, the formation of triose phosphate in the present experiment presumably was preceded by phosphorylation of fructose Phosphorylation of fructose by liver and kidney has been demonstrated (16)

The amount of inorganic phosphate that disappeared, 19.7 micromoles, corrected for phosphorylation attributable to lactate production, 4.4 micromoles, and for organic phosphate hydrolyzed by acid during 0 to 180 minutes, 2.4 micromoles, was 13.9 micromoles According to Equation 1, the oxygen uptake accounts for the disappearance of 5 micromoles of inorganic phosphate Experiments with glucose indicated a similar discrepancy between the phosphorylation predicted from the oxygen uptake and the observed disappearance of inorganic phosphate This discrepancy suggests the existence of additional unknown reactions

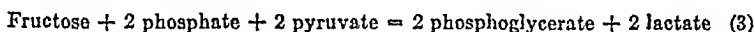
Experiment I, C, illustrates the effect of pyruvate on the aerobic oxidation of fructose In this experiment inorganic phosphate, oxygen, and carbonyl compounds disappeared, and organic phosphate and carbon dioxide were formed The rate of acid hydrolysis of the organic phosphate suggests that it was hexose phosphate In a large scale experiment the organic phosphate isolated as the barium salt contained fructose and phosphate in the ratio of about 2 and reduced the alkaline copper reagent This suggests that a considerable portion of the organic phosphate formed during the oxidation of the mixture of pyruvate and fructose was fructose diphosphate

The results may be attributed in part to the reaction described by the equation



The extent to which the phosphorylation observed in the present experiment can be attributed to the oxidation of pyruvate is uncertain The maximum correction of the phosphorylation observed in the presence of pyruvate plus fructose is probably represented by the sum of the disappearance of inorganic phosphate in the presence of fructose and pyruvate alone, 21.8 micromoles The corrected phosphorylation is 21.0 micromoles, giving a minimum ratio of *moles of inorganic phosphate esterified to moles of oxygen consumed* of 3.8 The ratio obtained with brain preparations with glucose as substrate was 4.0 (17) Experiments with preparations of cat heart indicate that the maximum ratio is probably 6 (18)

The anaerobic oxidation of fructose in the presence of pyruvate is illustrated by Experiment 2, A. Inorganic phosphate and pyruvate disappeared, lactate and organic phosphate were formed. The resistance of the organic phosphate to acid hydrolysis and the properties of the organic phosphate isolated in the large scale experiment suggest that the product of the oxidation of fructose was phosphoglycerate. An equation that accounts for the results is



This reaction, which has been demonstrated with brain preparations with glucose as substrate, involves dismutation between triose phosphate and pyruvate (15).

Aerobically in the absence of fluoride, fructose was converted to lactate. This is demonstrated by the data given in Experiment 3. The data are in agreement with the equation



The properties of the organic phosphate isolated in the large scale experiment were compatible with the assumption that the organic phosphate formed in Experiment 3 was mainly fructose diphosphate. With glucose as substrate, this reaction has been demonstrated with brain preparations (15).

With relatively concentrated preparations of brain little or no lactate was formed from glucose (15, 19) or fructose. It seemed possible that this may have been due to inactivation of the necessary diphosphopyridine nucleotide, which is effected by brain and which is prevented by nicotinamide (20, 21). However, the amide did not permit lactate formation from glucose or fructose by relatively concentrated preparations. It appears, therefore, that the failure of such preparations to produce lactate is not due to inactivation of the nucleotide. Previous attempts to demonstrate lactate formation from fructose in brain (3) probably did not succeed owing to the use of concentrated preparations.

SUMMARY

The oxidation of fructose by brain *in vitro* is accompanied by phosphorylation. The reactions that account in part for the oxidation of glucose and fructose are the same.

The laboratory is indebted to Merck and Company, Inc., for the thiamine pyrophosphate used in these experiments.

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SEDIMENTATION CONSTANTS AND ELECTROPHORETIC MOBILITIES OF ADULT AND FETAL CARBONYLHEMOGLOBIN

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Differences between the hemoglobin of the new born child and that of the adult have been recognized for some time. Following the observations of von Kruger (1, 2) that hemoglobin from different species is characterized by differences in rate of denaturation by alkali, Brinkman (3, 4), Haurowitz (5), and others (6, 7) have differentiated more than one hemoglobin in human blood by this means. At least two widely divergent types, a fetal and an adult type, and possibly others are present. The fetal, alkali-resistant type present at birth is gradually replaced during the first 7 months of life by an alkali-labile form. Preliminary studies by us have confirmed these observations. After 3 years of age, a small variable amount of alkali-resistant hemoglobin may recur. The latter has not been shown to be identical with the fetal type (3). Other differences in the properties of hemoglobin from individuals of different ages tend to substantiate the view that there is more than one hemoglobin. They differ in their affinity for oxygen and carbon dioxide (8-10), in crystalline form (11, 12), in amino acid content (13, 14), and in immunological specificity (15). According to Jungblood (16) they cannot, however, be distinguished spectrophotometrically. There appears to be a general agreement with Haurowitz (17) that the prosthetic moiety of the adult and fetal hemoglobin is identical and that the difference is in the protein fraction.

It was thought that the hemoglobins might be differentiated by electrophoretic mobilities and by their sedimentation constants, if the variation exists in the protein portion of the molecule. The electrophoretic mobilities and sedimentation constants of fetal and adult carbonylhemoglobin here reported show marked differences and demonstrate that two molecular species may be separated from fetal and adult blood.

EXPERIMENTAL

Our first studies of the mobility of hemoglobin were carried out on oxyhemoglobin which was later replaced by carbonylhemoglobin because of its greater stability.

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Citrated whole blood was saturated with carbon monoxide and centrifuged. The red cells were washed three times with 0.9 per cent sodium chloride saturated with carbon monoxide. Approximately 11 cc of the packed cells were transferred to a 50 cc centrifuge tube and 35 cc of water and 10 cc of toluene were added. After being refrigerated for several hours the tube was centrifuged and the clear carbonylhemoglobin solution filtered into a 100 cc flask, 11.5 cc of 0.2 M secondary sodium phosphate and 5.7 cc of 0.2 M primary sodium phosphate were added and the hemoglobin solution was made up to 100 cc with water. The concentration was determined electrophotometrically and adjusted to 0.5 per cent by the addition of a phosphate buffer consisting of 115.7 cc of 0.2 M secondary sodium phosphate and 57.8 cc of 0.2 M primary sodium phosphate per liter and saturated with carbon monoxide. This buffer, pH 7.1, was also used for the subsequent dialysis of the hemoglobin and in the Tiselius cell (18). The hemoglobin solution was dialyzed for 72 hours, after which time the conductivity of the buffer and that of the hemoglobin solution remained constant. Conductivity was measured at 4° by means of an audio frequency, alternating current bridge and an oscillator. A Longworth-Klett modification of the Tiselius apparatus was used. Pictures were taken initially and at suitable intervals by the schlieren method, by the Longworth scanning technique (19), or both, with a General Electric projector flood light as the light source and a red gelatin tricolor filter. Mobilities were calculated on the basis of the distance moved by the bisecting ordinate of the concentration gradient curve at 4°, pH 7.1, under the influence of a current of 0.020 ampere.

Values for mobility, u , were calculated from the distance moved, d , the conductivity, C , the current, i , cross-section of the cell, A , and the time in seconds, t , by the following formula, $u = CAd/i$. Carbonylhemoglobin for the determination of sedimentation constants was prepared according to the procedure of Steinhardt (20). The sedimentation constants were calculated by the Svedberg absorption technique (21) from data obtained by a McBain type ultracentrifuge (22). The 37 mm rotor used in this work is similar to all of the McBain rotors except that it is lower and more streamlined (23). The insert has the appearance of a heavy washer with sixteen radial holes drilled in it to hold small capillary tubes filled with the hemoglobin solution. The procedure is simply to put the filled and sealed capillary tubes into the rotor and, when a measurement is desired, to stop the rotor, remove the tube, photograph it, and replace in the rotor. This complete process is rapid with the McBain instrument, full speed being regained in about 2 minutes.

DISCUSSION

The results obtained on eleven specimens of carbonylhemoglobin have been summarized in Table I and Figs 1 to 3. There is a significant difference in the mobilities and in the sedimentation constants of adult and fetal

TABLE I
Electrophoretic Mobilities and Sedimentation Constants of Fetal and Adult Carbonylhemoglobin

Source of hemoglobin	0.5 per cent HbCO pH 7.1	Potential gra- dient 10 volts per sq. cm	Sedimentation constant $\frac{S}{20} \times 10^{-12}$
	Mobility ($\mu \times 10^{-5}$)		
	Slower compo- nent	Faster compo- nent	
Adult (2 samples)*	-0.7	(-1.0)	4.7 ³
5 day infant		-1.2	2.5 ⁵
9 " "	(-0.7)	-1.2	2.9 ⁵
90 " "	(-0.7)	-1.3	
Umbilical cord blood (2 samples)	(-0.8)	-1.3	
Mixture, umbilical cord and adult blood	(-0.7)	-1.3	

The values in parentheses were for small components.

* Three additional measurements of mobility were made on oxyhemoglobin with the same results.

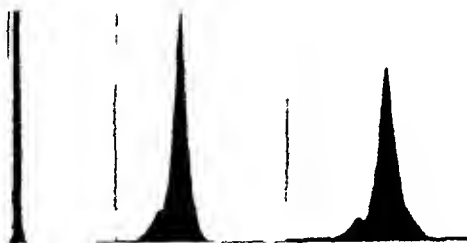


FIG 1 Longsworth electrophoretic pattern descending, of HbCO₂ derived from cord blood. The principal component, fetal hemoglobin, HbCO₂, is shown with the smaller component adult hemoglobin, HbCO₂, on the left appearing in 2 hours. The three peaks from left to right occur at 0, 2, and 4 hours.

hemoglobin. The blood from the umbilical cord and from 5, 9, and 90 day-old infants contains two hemoglobins, a large fraction with a characteristic electrophoretic mobility of fetal hemoglobin and a small fraction with the mobility of the hemoglobin obtained from adult blood. This finding agrees with the conclusions of Brinkman (4) and Haurowitz (5)

that the blood of a new born infant contains a small amount of adult hemoglobin. In one adult blood, a small fraction was noted, the mobility of which approximated that of the fetal type. The fraction was small and difficulty in measuring made the absolute mobility somewhat in doubt.

Marked differences in the sedimentation constants of the hemoglobin in the blood of two infants and one adult are evident. A measurement of molecular weight was not obtained, since diffusion constants were not

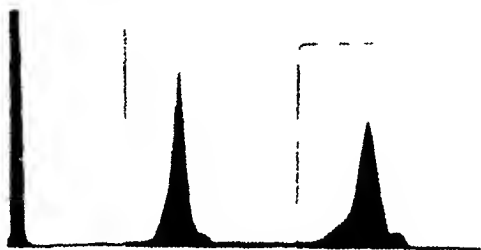


FIG 2 Longsworth electrophoretic pattern, descending, of HbCO_2 derived from adult blood. The principal component, HbCO_2 , and the small component on the right, HbCO , appearing in 2 hours are shown. The three peaks from left to right occur at 0, 2, and 4 hours.

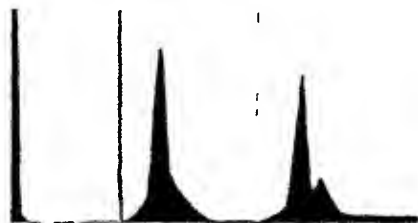


FIG 3 Longsworth electrophoretic pattern, descending, of equimolar mixtures of HbCO_2 and HbCO . The three peaks from left to right occur at 0, 2, and 3 hours. The small peak appearing on the right of the principal peak at 3 hours is the separating HbCO component.

determined. If one assumes the same diffusion constant, the molecular size of adult hemoglobin would be about twice that of the fetal hemoglobin.

These data substantiate the view that there are two molecular species of hemoglobin, fetal and adult, which differ in the protein portion of the molecule.

Nothing is known of the mechanism underlying the gradual appearance of an adult hemoglobin component during early life with the concomitant disappearance of the fetal fraction. The progressive decrease in hemoglobin values of blood which occurs during this same period may possibly be related to this change in mechanism.

SUMMARY

Electrophoretic mobilities and sedimentation constants of adult and fetal carbonylhemoglobins have been determined. From these data, it is concluded that adult and fetal hemoglobins consist of at least two molecular species, the relative proportions of which differ markedly in adult and fetal hemoglobin.

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A COLORIMETRIC DETERMINATION OF NICOTINIC ACID*

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(Received for publication, January 27, 1944)

Most chemical methods for the determination of nicotinic acid involve reaction with cyanogen bromide and a primary or secondary aromatic amine. Several different amines have been proposed, such as aniline (1), β -naphthylamine (2), *p*-methylanilinophenol sulfate (3), *p*-aminoacetophenone (4), *p*-aminophenol (5), *p*-aminophenylsulfonamide (6), and orthoform (methyl *m*-amino-*p*-hydroxybenzoate) (7). Such diversity is not without reason, since development of maximum color is dependent on the presence of the amine group. The difficulties most frequently met in the various methods are the necessity of waiting a certain length of time for development of the color, instability of the color, low color intensity, and failure adequately to account for the blank when turbid or colored extracts are being analyzed.

The difficulties mentioned above have been eliminated in the following method with *m*-phenylenediamine and hydrochloric acid.

EXPERIMENTAL

Acidification with hydrochloric acid stabilizes the maximum color developed by the reaction of nicotinic acid with cyanogen bromide and *m*-phenylenediamine. Color development is immediate and the intensity is greater than that obtained with many other amines.

Reagents—

1. A 5 per cent aqueous solution of *m*-phenylenediamine dihydrochloride, prepared freshly each week.
2. A 4 per cent aqueous solution of cyanogen bromide.
3. Buffer solution adjusted to pH 6.6 (7).
4. 20 per cent hydrochloric acid.
5. Standard nicotinic acid solution containing 1.0 mg per ml. When determinations are to be made, this solution is diluted so that each ml contains 10 γ of nicotinic acid.

Procedure

To 10 ml of a solution containing between 0 and 60 γ of nicotinic acid are added 5 ml of the buffer solution and 5 ml of the cyanogen bromide

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solution After the mixture is allowed to stand at room temperature for 20 minutes, 1 ml of the *m*-phenylenediamine solution is added, followed immediately by 1 ml of 20 per cent hydrochloric acid Readings are made in a photoelectric colorimeter (Klett-Summerson) at 400 $m\mu$ (Filter 40) The readings may be made immediately or at any time during the following 15 minutes In the preparation of a standard curve, the solution containing

TABLE I
Determinations with Pure Nicotinic Acid Solutions

Nicotinic acid (a)	Density scale reading (b)	$K, \frac{(a)}{(b)}$
γ		
0	0	
5	11	0.455
10	22	0.455
20	44	0.455
40	88	0.455
60	132	0.455

TABLE II
Effect of Acid on Stability of Color

The values are the actual readings on solutions containing 50 γ of nicotinic acid from the optical density scale of the colorimeter (distilled water, 0)

Time	No acid	1.0 ml 10 per cent HCl	1.0 ml 20 per cent HCl	1.0 ml concn trated HCl
min				
0.5	163	158	160	124
1	160	158	160	126
2	154	154	160	126
4	144	150	160	136
8	142	150	160	140
12	128	145	160	140
16	124	135	160	140
20	120	127	160	140
24	116	125	156	140
28	112	123	150	138
32	108	118	144	132

no nicotinic acid is used to set the zero point of the instrument, thus making scale readings directly proportional to the concentration of nicotinic acid, as shown in Table I K , the factor for converting optical density to concentration of nicotinic acid, should be determined each time analyses are carried out

Effect of HCl—Preliminary determinations with *m*-phenylenediamine

showed that maximum color develops within about 5 seconds at a pH between 6 and 7, but that it immediately begins to fade. However, stabilization of the color is effected for at least 15 minutes by acidification with 20 per cent hydrochloric acid. In Table II the effects on the stability of adding various concentrations of acid are shown. All samples contained 50 γ of nicotinic acid. The data are actual readings from the optical density scale of the colorimeter (distilled water, zero). Since addition of 1 ml of 20 per cent hydrochloric acid brings about stability of the color at a high intensity, this concentration has been chosen for the analytical procedure. The acid must be added immediately after the amine. Addition either before or with the amine (solution of the amine in 20 per cent hydrochloric acid) will result in a stable color but at a much reduced intensity.

Similar determinations were carried out with aniline and with orthoform substituted for *m*-phenylenediamine. With each of these amines maximum color development requires from 3 to 5 minutes and fading is rapid. An attempt to reproduce the stability which has been reported for the orthoform method (7) did not meet with success. Addition of hydrochloric acid resulted in total loss of color when orthoform was used, but produced color stability when aniline was the amine employed. However, the intensity of the color developed with aniline is only about half that produced with *m*-phenylenediamine, and so the latter is to be preferred. The larger number of amine groups present may account for this increased color intensity. Such reasoning is in keeping with the statement by König (8) that by increasing the concentration of the amine an increasing number of groups are forced into combination.

Blank.—In the analysis of biological materials, foods, and pharmaceuticals, it is often difficult to obtain a solution completely free from color or turbidity. Hence, correct allowance for such factors is essential. The use of an extrapolation method produces false values according to Dann and Handler (9), and so a true blank determination should be made. However, the usual type of blank is not sufficient, because, as shown by Melnick and Field (10), the amine reacts with substances in extracts to produce a color, but this reaction does not occur when cyanogen bromide is present. This necessitates omitting both the amine and cyanogen bromide from the blank when the color due to the extract is being determined, and requires the measurement of two blanks, the effects of which are additive. One of these is termed the sample blank and consists of 10 ml of the extract being assayed, 5 ml of buffer solution, 6 ml of water, and 1 ml of 20 per cent hydrochloric acid. A distinct advantage of the acidification enters at this stage of the procedure, since any protein in the extract which is precipitated by the alcohol of the buffer is immediately redissolved upon addition of hydrochloric acid, thus providing a clear yellow solution.

Thus, of course, applies to the sample being assayed as well as to the sample blank. The second blank is termed the reagent blank and consists of 16 ml of water, 5 ml of cyanogen bromide solution, and 1 ml of the 5 per cent *m*-phenylenediamine solution. This latter blank is constant and need be determined only once for a series of assays. However, it is not possible to set the colorimeter at zero for the reagent blank, because the reagents often produce more color than is present in the sample blank. Therefore, it is best to set the instrument at zero for some arbitrary point (distilled water) and, in making an assay, obtain the true reading, which is propor

TABLE III
Determinations with Commercial Vitamin Tablets

Sample No	Nicotinic acid added per tablet	Nicotinic acid found per tablet	Recovery of added nicotinic acid
	mg	mg	per cent
1	0 00	4 72	
2	0 00	4 72	
3	1 00	5 72	100 0
4	1 00	5 72	100 0

TABLE IV
Determinations with Blood (Cow)

Sample No	Nicotinic acid added per 100 ml	Nicotinic acid found per 100 ml	Recovery of added nicotinic acid
	mg	mg	per cent
1	0 00	0 41	
2	0 00	0 41	
3	1 00	1 41	100 0
4	1 00	1 37	97 2
5	2 00	2 41	100 0
6	2 00	2 46	102 0
7	3 00	3 55	104 2
8	3 00	3 50	102 6

tional to the concentration of nicotinic acid, by subtracting from the sample reading the sum of the readings of the two blanks.

In order to test the validity of the dual blank procedure, recovery experiments were carried out with blood and with commercial vitamin tablets, both of which substances yielded colored extracts. The blood samples were extracted according to the method of Friedemann and Barboraka (11), while the vitamin tablets were extracted with hot water, centrifuged, and the centrifugate diluted until 10 ml contained between 10 and 50 γ of nicotinic acid. The data, presented in Tables III and IV, substantiate the accuracy of the procedure with the blanks.

SUMMARY

The use of *m*-phenylenediamine and 20 per cent hydrochloric acid in the colorimetric determination of nicotinic acid has been described. The reaction is immediate and produces a color which has a fairly high optical density and which is stable for over 15 minutes.

A method of calculation, involving dual blanks, for use when colored or turbid extracts are being assayed has also been presented.

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THE HYDROLYSIS OF CYSTINE AND THE FRACTIONATION OF SULFUR IN PLANT TISSUES

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It is generally recognized that when biological material is subjected to alkaline hydrolysis for the liberation of sulfide sulfur, the principal source of the sulfur is cystine or closely related substances. This sulfur is often called "labile" sulfur. The older methods (7, 8) for estimating "labile" sulfur were quite arbitrary and were usually intended to distinguish between more or less loosely bound and firmly bound sulfur rather than to estimate the sulfur in any particular compound, though it was understood that one-half to three-fourths of the cystine sulfur might be so recovered, and it was suggested (10) that glutathione might be the source of the most readily hydrolyzed sulfur.

Clarke and Inouye (4) found that the sodium plumbite hydrolysis of cysteine gave a quantitative yield of lead sulfide but that cystine yielded only 75 to 80 per cent of its sulfur as lead sulfide. The latter was removed, oxidized, and determined gravimetrically. The remainder of the sulfur was assumed to be in the form of thiosulfate. Ambler (1), using cystine standards and rigidly controlled digestion with lead acetate and magnesium oxide, was able to obtain reproducible "labile" sulfur values in sugar solutions, which he expressed as the equivalent amounts of cystine.

In the present work the disposition of the sulfur after hydrolysis of cystine by a mild alkaline digestion has been studied and the treatment has been applied to the fractionation of the sulfur in plant material.

Determination of "Labile" Sulfur

Apparatus—The apparatus of Ambler (1) was modified as shown in Fig. 1. Dropping funnels were provided so that the contents of the digestion flask could be acidified either through the intake tube or through the condenser. The absorbers were 125 ml bottles with ground glass stoppers, but rubber stoppers were used in the assembly line. Hydrogen (or nitrogen) free of oxygen was passed slowly through the apparatus. A 3-way stop-cock or a glass T before each absorber permitted addition of reagents to the absorber without exposing its contents to the air. Four units of apparatus were operated simultaneously from one source of hydrogen. In some of the analyses, the digestion mixture was filtered in order

to treat the precipitated sulfide independently of the other products of the reaction. This was accomplished by removing the intake dropping funnel and the absorbers and connecting the filtering apparatus, as shown by the broken lines in Fig 1. By proper manipulation of the stop-cocks, the suspension could be drawn from the flask and filtered under hydrogen.

Digestion—The base used throughout the work was magnesium oxide. A few tests with leaf tissue indicated that the use of 0.5 N or 4.0 N potas

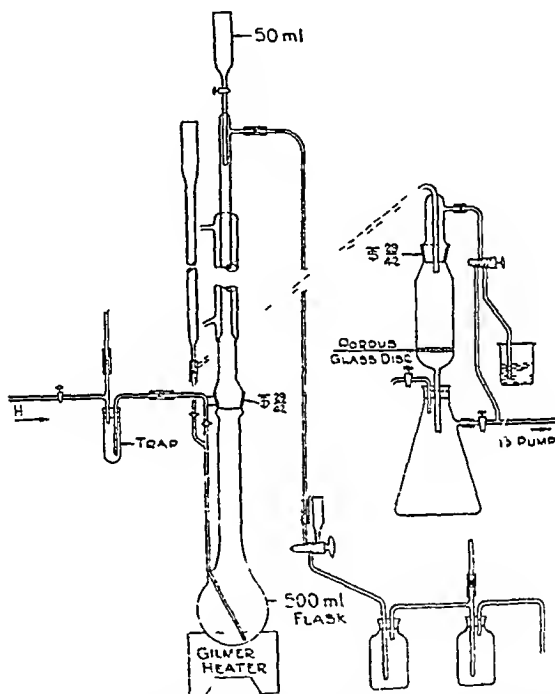


FIG 1 Apparatus for alkaline digestion of cystine or biological material and subsequent evolution of hydrogen sulfide, etc., or filtration under hydrogen of cadmium sulfide

sium hydroxide did not increase the yield of sulfide. In view of the fact that the latter caused troublesome foaming, magnesia was preferred. A number of heavy metals were added to the reaction mixture containing leaf tissue in an effort to find the best collector of the hydrolyzed sulfide. Digestion with cadmium chloride or hydroxide gave the maximum yield. Bismuth and lead salts gave about 85 to 90 per cent of the yield obtained with cadmium, zinc salts about 65 per cent, silver and mercury salts about

50 per cent, cobalt, nickel, and stannous salts about 30 to 40 per cent, antimony, manganese, cuprous and cupric salts only 0 to 3 per cent. If no collector was used and the hydrogen sulfide was swept out continuously as rapidly as it was formed, a yield of 75 per cent of sulfide was obtained. Cadmium salts also gave a higher yield than lead in the hydrolysis of pure cystine and they had an additional advantage over lead for our purpose in that they did not interfere in the analysis of the reaction mixture for the sulfur remaining after the removal of the sulfide.

The leaf tissue studied was generally fresh material kept frozen in tight containers at -15° . 15 gm portions were comminuted in a Waring blender. The suspension was diluted with water to 600 ml and while being thoroughly agitated two 200 ml portions were taken for sulfide analysis and three 50 ml portions for dry weight determination. Leaf samples which had been rapidly air-dried by being spread out in a warm room gave results comparable with the frozen material. 1 or 2 gm portions of the air-dry leaves were used.

The samples of pure chemicals or leaf tissue were mixed with 25 ml of a 5 per cent suspension of magnesia and 1 gm of cadmium chloride and diluted to 325 ml. The apparatus was flushed out with hydrogen before the flask was heated and the digestion was allowed to proceed with gentle boiling in a very slow stream of hydrogen. Two Gilmer heaters in series supplied the correct amount of heat. The flask was covered with a hood of asbestos paper to exclude the light. The digestion period varied with the material. The hydrolysis of thiourea was complete in 1 hour or less, cystine required 12 to 20 hours, casein, 1 to 2 days, depending on the size of the sample, and leaf tissue, 2 to 4 days.

Absorption and Estimation of Sulfide and Sulfite—In one procedure the reaction mixture was acidified with sufficient boiling approximately normal, hydrochloric acid to dissolve the magnesia and cadmium sulfide. It was best to introduce the acid through the intake tube, but the condenser sometimes needed washing. A stream of hydrogen removed the hydrogen sulfide and also some sulfur dioxide as rapidly as they were liberated. Absorption was accomplished in two absorbers each containing 10 ml of St. Lorient's (1) solution (5 per cent zinc acetate, 1 per cent sodium acetate, and 0.5 per cent sodium chloride) diluted with 50 ml of water. In some experiments the zinc absorbers were preceded by an absorber containing 50 ml of phosphate-borate buffer (2 per cent disodium phosphate crystals and 0.8 per cent boric acid). This solution had a pH of 7.6 to 7.7 and served effectively to separate small amounts of sulfur dioxide and hydrogen sulfide.

Estimation of the absorbed sulfur compounds was completed by one of three methods. First, by converting the sulfide to methylene blue (1),

or second, iodometrically in acid solution, or third, bromometrically by the Manchot and Oberhauser method (6), followed by precipitation of barium sulfate. The methylene blue method is specific for hydrogen sulfide, but it was difficult to control the reactions involved so as to secure quantitative results. The iodometric analysis was carried out by adding potassium iodide first, then an acid solution of 0.015 N iodine or iodate, followed by titration with 0.01 N thiosulfate. This order of adding the reagents was necessary to prevent overconsumption of iodine by the larger amounts of sulfide. Sulfur dioxide has the same iodine factor as hydrogen sulfide and the titration will, therefore, give total sulfur correctly even if both forms are present in a mixture.

For the bromometric titrations, solutions of 0.1 and 0.02 N bromine in normal potassium bromide were used. It was necessary to add an excess of about 10 ml. of 0.1 N bromine in a total volume of 100 ml. in order to oxidize sulfide to sulfate in a few minutes. Only a slight excess of bromine was necessary for the sulfite oxidation. A little acid was added after the bromine, and the bottle was closed with a glass stopper with a minimum exposure of the bromine solution. The excess bromine was titrated with standard 0.1 N arsenious acid until the solution was colorless, then a drop of methyl red was added, and 0.02 N bromine was added drop by drop until the indicator was suddenly decolorized. It was helpful in titrating the bromine to replace the glass stopper with another glass stopper having a small hole drilled through it to accommodate the burette tip. Barium chloride was then added to the solution for a gravimetric sulfate determination. Since sulfide requires 4 times as much bromine for oxidation as sulfite, the bromometric method in conjunction with the gravimetric served to identify qualitatively as well as to determine quantitatively the two sulfur forms. The principal objection to this method arose from the fact that bromine oxidized some volatile organic compounds, which could not be excluded completely. The iodometric method was not subject to this objection to an appreciable extent.

Hydrolysis of Cystine—The hydrolysis of cystine was studied with an Eastman preparation both as supplied and after repeated recrystallization. The purity was determined by sulfur analysis in the Parr bomb. Table I summarizes a series of experiments in which various amounts of cystine were digested for different lengths of time. The products of the hydrolysis were collected in the zinc acetate absorbing solution and titrated iodometrically. The data indicate that the reaction was not complete in 4 hours but that the yield of volatile sulfur compounds was constant at 89 to 96 per cent of the total sulfur after 12 to 68 hours with amounts of cystine up to 20 mg. Protracted digestion evidently did no harm.

Table I also shows that small amounts of cysteine, thiourea, and thio-

semicarbazide gave quantitative yields of sulfur by this method. It seems probable that these substances give higher yields than cystine because in them only a simple hydrolysis is involved.

A number of other sulfur compounds, notably methionine, gave negative results, and only 0.7 per cent of the sulfur in thioglycolic acid was hydrolyzed in 2 days. A commercial casein preparation yielded 0.085 per cent

TABLE I

Hydrolysis of Cystine and Some Other Sulfur Compounds, and Iodometric Determination of Volatile Products of Hydrolysis after Acidification

Substance	Weight of sample	Weight of sulfur	Time of digestion	Sulfur recovered		
				mg	per cent	Average per cent
Cystine	5.0	1.33	4	1.12	84	
	10.0	2.66	12	2.36	89	
	2.5	0.65	20	0.59	91	
	5.0	1.30	20	1.21	93	
	7.5	1.95	20	1.80	92	
	10.0	2.60	20	2.36	91	
	15.0	3.90	20	3.68	94	
	20.0	5.32	20	4.85	91	92
	2.5	0.65	44	0.59	91	
	5.0	1.30	44	1.20	92	
	10.0	2.60	44	2.37	91	
	15.0	3.90	44	3.67	94	92
	2.5	0.65	68	0.62	96	
	5.0	1.30	68	1.15	89	
	10.0	2.60	68	2.39	92	
	15.0	3.90	68	3.64	93	92
Cysteine hydrochloride	5.0	0.92	18	0.93	101	
	10.0	1.84	18	1.86	101	
	20.0	3.68	18	3.74	102	101
Thiosemicarbazide	6.25	2.13	2	2.12	100	
	6.25	2.13	19	2.23	105	102
Thiourea	5.0	2.10	1	2.12	101	
	5.0	2.10	3	2.09	100	
	5.0	2.10	12	2.14	102	101

sulfur in a number of concordant analyses. This is about the same as the "labile" sulfur found by Clarke and his associates (3, 12) and may be compared with the value 0.075 to 0.08 per cent for cystine sulfur reported by Sullivan (9) and 0.08 per cent reported by Baernstein (2).

In another series of experiments it was found that the sulfur recovery from cystine by iodometric titration was 90 ± 2 per cent, whereas by the methylene blue method the recovery was 83 ± 6 per cent. This suggested

Examination of the filtrates from these reactions by the methods of Kurt-enacker and Goldbach (5) revealed no more than traces of thiosulfate or polythionates

The third treatment (C) involved filtration under hydrogen and direct oxidation of the sulfide to sulfate by bromine. This oxidation could not be estimated bromometrically because organic matter was present which

TABLE III

Sulfur Dioxide, Hydrogen Sulfide, and Sulfate Determined Gravimetrically after Hydrolysis of Cystine, Cysteine, and Glutathione

	No of analyses	Treat ment	Weight of sample	Weight of S	Sulfur recovered as			
					Sulfide	Sulfite	Sulfate	Total
			mg	mg	per cent	per cent	per cent	per cent
Cystine	2	A	5	1 33	78	19	†	97+
	4	"	10	2 66	77	17	6	100
	7	"	20	5 32	74	17	†	91+
	3	"	25	6 65	76	11	†	88+
	3	"	50	13 30	72	8	4	84
	2	"	100	26 60	66	6	8	80
	2	B	50	13 0	76	6	6	88
	2	"	100	26 0	77	4	8	89
	2	C	10	2 66	100		2	102
	4	"	50	13 2	94		5	99
	3	"	100	26 3	92		7	99
	1	A	5	0 92	101	3	†	104
Cysteine	3	"	10	1 84	99	6	†	105
	3	"	25	4 60	87	3	†	90
	2†	"	25	4 60	97	2	†	99
	1	"	50	7 65	73	1	†	74
Glutathione	2	"	10	1 04	103	13	†	116
	2	"	25	2 60	90	5	†	95

* Treatment A, acidify and sweep with hydrogen. Treatment B, filter CdS under H₂, return precipitate to flask, acidify, and sweep with H₂. Treatment C, filter CdS under H₂ and oxidize CdS with Br water

† Not determined

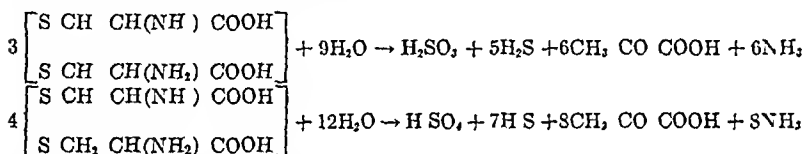
‡ 1 to 2 gm. of mannitol added to digestion mixture

caused overconsumption of bromine. Practically all the sulfur was accounted for as sulfide and sulfate. Only a trace of sulfur dioxide could be evolved from the filtrate but any sulfite precipitated with the sulfide would be included with the latter. In other experiments with 1.0 gm samples of cystine, after 2 days digestion, 87.5 per cent of the sulfur was recovered as sulfide, 9.2 per cent as sulfate, 1.5 per cent as base-soluble organic sulfur, and 1.8 per cent as base-insoluble, acid-soluble, organic sulfur. The last

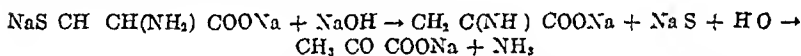
item probably came partly from unchanged cystine deposited in the intake tube and protected from the digestion mixture

DISCUSSION

A detailed study of the reaction products of the cystine hydrolysis is being made¹ the results of which will be published later. At this time it may be indicated (1) that pyruvic acid has been isolated after the completion of the digestion as one of the principal reaction products, without addition of a precipitating agent to the mixture during digestion as was done by Clarke and Inouye (4), (2) attempts to isolate glyceric acid have been unsuccessful, (3) sparingly soluble organic substances are present in the MgO-CdO-CdS mixture, and (4) deamination and evolution of ammonia are nearly complete during the digestion. The following over-all reactions therefore suggest themselves



Bergmann and also Clarke and Inouye (4) have suggested a mechanism for conversion of cystine to pyruvic acid



Without entering into a detailed consideration of the process, we may say that the splitting of the disulfide linkage appears fairly satisfactorily explained by oxidation of part of the sulfur to sulfite and sulfate. The equations require that the sum of sulfite and sulfate sulfur shall be between 12.5 and 16.7 per cent of the total. The values in Table III indicate that this condition is approximately fulfilled. With small samples of cystine the values are slightly high and with large samples slightly low. Sulfite values as found by Treatment B are probably included in the sulfide values by Treatment C. With such an allowance the equations are nearly exactly fulfilled in the case of the 1.0 gm samples of cystine. It may be noted that a very small amount of sulfite is derived from cystine, suggesting that other reactions are not entirely excluded. The separation of free sulfur has not been observed (4). It should be emphasized that in the present hydrolysis the pH of the reaction mixture was about 8.5. Less drastic reactions can therefore be expected than with stronger alkali.

¹ The authors are indebted to A. J. Johanson, Brigham Young University, Provo, Utah, for assistance with this study.

It also appears that the deamination and production of pyruvic acid can be accomplished by hydrolytic or compensated oxidation-reduction reactions without addition of an oxidizing agent to the system. Pyruvic acid has long been known to react readily with hydrogen sulfide to form thioldilactic acid. This fact may explain the reduced yields of hydrogen sulfide by acid evolution from the digestion of the larger samples of cystine. Owing to concentration effects, the proportion of hydrogen sulfide thus fixed would probably increase as the weight of cystine digested increased. In a special experiment, a mixture of sodium sulfide and pyruvic acid evolved appreciably less hydrogen sulfide than did sodium sulfide alone. In the

TABLE IV
Sulfur Fractionation of Sample of Alfalfa Leaves

Treatment ^a	Sulfur recovered							
	H S + SO ₂	CdS	SO ₂	Organic sulfur				Total
				Base soluble	Base insoluble		Total soluble	
					Acid soluble	Acid insoluble		
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
A	0 096		0 233			0 106	0 150	0 535
B	0 097		0 226	0 087	0 057	0 111	0 144	0 578
C		0 095† 0 018‡	0 218	0 076	0 043† 0 033‡	0 95	0 152	0 578

Total sulfur by bomb 0.57 per cent

* After 3 to 4 days digestion. Treatment A, acidify and sweep with H₂; Treatment B, filter CdS under H₂, return precipitate to flask, acidify, and sweep with hydrogen; Treatment C, filter CdS under H₂, and treat precipitate successively with cold and hot Br water. Analyze all filtrates for sulfate and organic sulfur.

† Digest with cold Br water.

‡ Digest with hot Br water.

case of Treatment B in Table III, the organic material in the MgO CdO CdS mixture probably interfered similarly with hydrogen sulfide evolution after acidification but to a smaller extent. It may be noted that Clarke and Inouye (4) were able to recover only 86.5 to 90.5 per cent of the sulfur in all inorganic fractions when they used a technique analogous to Treatment B for "labile" sulfur analysis of 1.0 gm. samples of cystine.

Hydrolysis of Leaf Tissue—Sulfur distribution of a single sample of powdered alfalfa leaves is summarized in Table IV. The three treatments employed in Table III were applied to the leaf tissue after a digestion period of 3 days. About 5 per cent of the sulfur evolved after acidification came off as sulfur dioxide. The "labile" sulfur values by the three methods

agreed satisfactorily, except that when the cadmium sulfide precipitate was given a vigorous treatment with hot bromine water high results were obtained, owing presumably to the oxidation of organic sulfur compounds precipitated with sulfide. Similarly, the three methods yielded nearly the same amount of sulfate.

The filtrates, after removal of sulfate, were evaporated to dryness in large nickel crucibles. The solutions were rendered alkaline during the evaporation. The residues were fused with sodium peroxide to destroy the organic matter, and the sulfate formed was determined gravimetrically to give soluble organic sulfur. In the second and third methods, the soluble

TABLE V

Comparison of Iodometric and Gravimetric Analyses for "Labile" Sulfur in Alfalfa Leaves

Crop (1939)	Cultural treatment			Sulfide S		
	SO ₂ fumigation	Sulfur level in	pH in	Iodometric		Gravimetric
		Nutrient solution		Fresh frozen sample	Air dry sample	Air-dry sample
				per cent	per cent	per cent
1st	None	Low	Low	0.115	0.112	0.107*
1st	"	"	High	0.107	0.103	0.097*
1st	Fumigated†	High	"	0.128	0.122	0.122*
1st	"	Low	Low	0.132	0.122	0.126*
4th	None	"	"	0.075		0.064‡
4th	"	"	High	0.076		0.066‡
4th	Fumigated§	"	Low	0.141		0.128‡
4th	"	"	High	0.132		0.118‡

* Acidify and sweep with H₂

† 293 hours at 0.11 part per million of SO₂

‡ Filter CdS and treat with Br water

§ 223 hours at 0.09 part per million of SO₂

organic sulfur was about equally divided between the two filtrates, obtained before and after treatment of the cadmium sulfide. These fractions probably include the methionine sulfur, since preliminary experiments indicate that methionine forms a sparingly soluble magnesium salt, a considerable part of which is retained in the base-insoluble residue, the remainder passing into the filtrate. The behavior of methionine is being investigated further.

In addition to these fractions, some insoluble organic matter, consisting of cell wall material and complex organic compounds, was present in the reaction mixture, after solution of the cadmium sulfide. The sulfur in this material was determined by the Parr bomb method. The first and

second methods gave practically the same results for this fraction, but Treatment C gave slightly low results, owing probably to the vigorous bromine treatment. It may be noted that this sample lost 0.01 per cent of "labile" sulfur and gained 0.01 per cent of acid-insoluble organic sulfur as a result of 3 years storage in a cool dark place.

In Table V, eight samples of fresh alfalfa leaf tissue were analyzed iodometrically for "labile" sulfur a few days after harvest, by the technique described earlier. The digestion time was 3 days. Aliquot portions of these samples were air-dried and 6 to 9 months later the aliquots were analyzed iodometrically and gravimetrically. The different determinations gave approximately the same results, but the results with the air-dried samples were a little lower than with the fresh samples. This alfalfa was grown in sand culture and all the plots except one had an inadequate supply of sulfate sulfur in the nutrient solution (11). It may be noted that the sulfur-deficient unfumigated plots had considerably less "labile" sulfur than the corresponding "fumigated" plots, particularly on the fourth crop, when the sulfur deficiency was more marked than on the first crop. Evidently fumigation with sublethal concentrations of sulfur dioxide raised appreciably the "labile" sulfur level of alfalfa growing in a low sulfate nutrient solution. The reproducibility of the results is satisfactory. The "labile" sulfur values should be increased by 10 per cent to express them as the equivalent amounts of cystine.

SUMMARY

Analysis of cystine by mild alkaline digestion in the presence of a cadmium salt, followed by acidification and evolution of hydrogen sulfide in the reaction flask or by filtration and oxidation of the cadmium sulfide, has been studied with many variations of the procedure.

1. Cadmium salt is the most efficient collector of sulfide.
2. Hydrolysis of cystine is nearly complete in 12 to 20 hours.
3. After acidification and depending on the weight of cystine digested, 66 to 78 per cent of the sulfur is evolved as sulfide, 6 to 19 per cent as sulfur dioxide, and 4 to 9 per cent is oxidized to sulfate. Lower yields with larger samples are probably due to formation of stable organic compounds during the evolution process.
4. Iodometric titration of the evolved gases is a convenient and accurate method of analysis. The results agree well with bromometric and gravimetric methods.
5. When the cadmium sulfide is filtered off and oxidized, nearly all the sulfur is accounted for as sulfide (including sulfite) and sulfate even with larger samples of cystine.
6. The amount of sulfite and sulfate produced in the digestion is practically equivalent to the reduction of the disulfide linkage.

7 Hydrolytic reactions account for the deamination of the cystine and also the production of sulfide and pyruvic acid

8 The digestion has been applied to leaf tissue and the sulfur has been partitioned into "labile" or "cystine" sulfur, sulfate, soluble organic sulfur, and insoluble organic sulfur The results are reproducible

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A QUANTITATIVE STUDY OF THE ADSORPTION OF ESTRONE, ESTRIOL, AND α -ESTRADIOL ON A CHROMATOGRAPHIC COLUMN

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In the course of a study of various procedures for the concentration and purification of urinary estrogens, the feasibility of the chromatographic technique seemed to warrant investigation. Chromatography has been employed to advantage in isolation procedures (1-3) for certain estrogens but no attempt has been made to utilize it in any quantitative procedure for estrone, estriol, and α -estradiol, the three estrogens believed to occur in human female urine (4, 5). Furthermore, it was suspected that sufficient differences in adsorptive characteristics might exist among the three estrogens to effect a reasonably accurate separation from a mixture. Current procedures for the quantitative separation of mixtures of the three naturally occurring estrogens depend upon differences in distribution between immiscible solvents (6-8) or upon differences in chemical reactivity of the C-17 substituent group (9, 10).

We have investigated the adsorption characteristics of crystalline estrone, estriol, and α -estradiol on a column of activated alumina and have developed a chromatographic procedure for the quantitative separation of the strongly phenolic estrogen (estriol) from the weakly phenolic estrogens (estrone and α -estradiol). This investigation and procedure form the subject of this report. The application of the chromatographic technique to suitable urinary estrogenic residues will be the subject of a forthcoming publication.

EXPERIMENTAL

Reagents—

Standard solutions of estrogens. Two weighed samples of crystalline estrone, two of estriol, and two of α -estradiol¹ were taken up in 95 per cent alcohol. Suitable aliquots of these solutions (containing from 25 to 300 γ) were transferred to test-tubes, and the solvent then evaporated by immersing the tubes in a boiling water bath. The residues left after evaporation were dried *in vacuo* overnight.

¹ Supplies of crystalline estrone, estriol, and α -estradiol were generously supplied by Dr. Erwin Schwenk of the Schering Corporation, estrone and estriol by Dr. O. Kamm of Parke, Davis and Company, and α -estradiol by Dr. Ernst Oppenheimer of the Ciba Pharmaceutical Products, Inc.

Kober's reagent prepared and employed essentially as described by Venning *et al* (11). A calibration curve for each estrogen was employed to convert color density at 520 $m\mu$ into micrograms of estrogen, since each exhibits a significant difference in this respect.

Zimmermann's reagent (12) was prepared and employed as described by Talbot *et al* (13).

The David test for estriol (14) was performed essentially as described by Pincus *et al* (15) except that 6 ml of water were added instead of 2.6 ml in order to permit readings to be made on the Evelyn photoelectric colorimeter. With a 620 $m\mu$ filter, Beer's law holds for estriol over the range 10 to 60 γ . $K = 0.052$.

Benzene (Baker's c.p., thiophene-free) was dried over calcium chloride and distilled in an all-glass apparatus.

Methyl alcohol, absolute, Merck reagent.

Ethyl alcohol, absolute. United States Industrial Chemicals, Inc., 200 proof.

Aluminum oxide, anhydrous, Merck, according to Brockmann (Merck and Company, Inc.) screened to pass a 200 mesh sieve.

Apparatus—The adsorption tube employed was 19 mm by 200 mm. The middle section was made from a distilling vacuum adapter² the upper male joint of which was sealed by a fritted glass disk. The bottom section consisted of interchangeable flasks with ground glass joints. A negative pressure from a water aspirator was used to maintain the proper rate of flow.

The Evelyn photoelectric colorimeter was used throughout, measurements being made with the 520 and 620 $m\mu$ filters.

Procedure

Preparation of Liquid Chromatogram (16)—30 gm of anhydrous aluminum oxide were added in small portions to a well dried chromatographic tube and gently tamped after each 1 to 2 cm rise in height. Employment of a negative pressure after the first addition of aluminum oxide prevented any tendency for it to creep up the side of the tube during tamping. A freshly prepared column was used for each determination.

The appropriate estrogen residue was then dissolved in 0.5 ml of absolute ethanol and diluted with 30 ml of benzene. The solution and each eluent were then added to the column from a small separatory funnel and a negative pressure applied so as to regulate the flow of the percolate to approximately 25 ml per 10 minute interval. Each successive 25 ml portion of eluent was added to the column when the meniscus of the preceding portion fell to within 0.5 cm of the top of the column, at which time the filtrate receptacle was replaced by a fresh one.

² Scientific Glass Apparatus Company, Catalogue 140, No J-1516.

The small amount of filtrate from the hormone solution itself was always included in the filtrate from the first 25 ml portion of the succeeding eluent. The solvent was then distilled from each fraction of the filtrate and the hormone residue or an aliquot part was transferred to a colorimeter tube with a small amount of ethyl alcohol. The tube was prepared for colorimetry by evaporating the alcohol and storing the residue *in vacuo* for 24 hours.

Liquid chromatograms for each individual estrogen were prepared with a limited variety of eluents. The behavior of each estrogen to a succession of solvents having increasingly powerful eluent actions was then investigated. Finally the behavior of a mixture of estrogens to such a succession of eluents was tested. From these data a suitable quantitative procedure for separating mixtures of the three estrogens was evolved and tested with varying relative proportions of estrogen mixtures. To determine the quantitative features of the method each eluent was collected *in toto*, rather than in 25 ml fractions, and tested for estrogen content.

Results

Benzene appeared to be the most suitable choice of solvent for the three estrogens. In view of the low solubility of estriol in benzene (17) a very small amount (0.5 ml) of absolute alcohol seemed desirable to aid in dissolving the estrogen residues. Consequently each estrogen residue for chromatography was taken up in 0.5 ml of absolute alcohol and diluted with 30 ml of benzene.

A series of determinations on the extent of development permissible with pure benzene in the case of each estrogen was carried out. Table I contains a summary of our results. It can be seen that development with as much as 225 ml of benzene failed to elute any detectable amount of any one of the three estrogens.

A number of attempts at extrusion and division of the aluminum oxide columns proved difficult and suggests the infeasibility of this method of separation. However, it was possible by this method to demonstrate that the three estrogens were adsorbed in the top third of the column.

The liquid chromatogram (16) technique was then adopted and a study was made of a number of solvents which might serve as suitable eluents. A summary of the effectiveness of the most suitable solvents in the series investigated is shown in Table I. It is apparent that admixture of small proportions of absolute methanol or ethanol with benzene provides a suitable eluent. Furthermore, it appears that absolute methanol is more effective, volume for volume, than absolute ethanol, especially in the case of estriol, which is more strongly adsorbed than the weakly phenolic estrogens (estrone and α -estradiol). It is also apparent that estrone and α -estradiol are strikingly similar in adsorptive power on activated alumina with respect

to the eluents listed and consequently offer little hope for separation. However, these eluents do seem to be sufficiently selective in effect to accomplish the separation of estrinol from mixtures containing the other two estrogens.

In order to develop a suitable scheme of successive eluents it is necessary to know how each estrogen distributes itself in such a scheme and how it is affected by the presence of other components of the mixture. Table II gives a summary of a number of determinations which seemed most likely to serve as a basis for a quantitative separation of estrinol from estrone and α -estradiol. 75 ml of pure benzene were chosen as a convenient amount of developing fluid. Then 100 ml of benzene-methanol mixture

TABLE I

Distribution of Estrogens Eluted from Chromatographic Column of Activated Alumina by Successive 25 Ml Washings of Various Eluents

Experiment No	Estrogen, 200-300 γ	Eluent	Per cent of total eluate in each washing*							
			2nd	3rd	4th	5th	6th	7th	8th	9th
1	Estrone	Benzene	0	0	0	0	0	0	0	0
2	"	Benzene-ethanol (9 1)	70	24	6	0	0	0	0	0
3	"	Benzene-methanol (9 1)	89	6	5	0	0	0	0	0
4	α -Estradiol	Benzene	0	0	0	0	0	0	0	0
5	"	Benzene-ethanol (9 1)	17	61	12	4	3	0	0	0
6	"	Benzene-methanol (9 1)	5	71	19	5	0	0	0	0
7	Estrinol	Benzene	0	0	0	0	0	0	0	0
8	"	Benzene-ethanol (9 1)	0	0	0	0	0	0	0	0
9	"	Benzene-methanol (9 1)	0	0	0	0	46	23	15	11
10	"	Benzene-ethanol (4 1)	0	0	0	0	0	2	12	
11	"	Benzene-methanol (4 1)	4	26	45	15	6	4	0	0

* Determined by the Kober reagent. The results were negative throughout for the first washing with 25 ml.

(9 1) and 125 ml of benzene-methanol mixture (4 1) were added in successive 25 ml portions. In the case of estrone and α -estradiol there is again a very marked similarity in the percentage distribution in the various fractions. However, estrinol is sufficiently dispersed from these two estrogens to permit nearly quantitative separation.

As a final test of the feasibility and dependability of the procedure, various binary mixtures were tested and recoveries of added amounts of estrogens determined colorimetrically. The data are summarized in Table III. The liquid chromatograms of single estrogens in the amounts indicated reveal that the procedure is adequately specific in that no detectable (*i.e.*, colorimetrically) amount of estrone or α -estradiol appears outside the 9 1 benzene-methanol eluate. On the other hand, no detectable amount

TABLE II

Distribution of Estrogens Eluted from Chromatographic Column of Activated Alumina by Successive Washings with Solvents Possessing Increasingly Powerful Eluent Actions

The figures refer to the percentage of the total amount recovered in each solvent

Experiment No	Amount of estrogen added	Color reagent used	Solvent* in successive 25 ml portions							
			Benzene methanol (9 1)			Benzene methanol (4 1)				
			2nd	3rd	4th	1st	2nd	3rd	4th	5th
1	200 γ estrone	Kober	87	9	4	0	0	0	0	0
2	200 " estriol	"	0	0	0	6	55	21	12	6
3	300 " α -estradiol	"	20	71	9	0	0	0	0	0
4	200 " estrone	Zimmermann	88	9	3	0	0	0	0	0
	200 " estriol	David	0	0	0	4	73	12	6	5
5	200 " "	"	0	0	0	6	58	23	8	5
	300 " α -estradiol	Kober	18	71	11					
6	200 " estrone	Zimmermann	86	14	0	0	0	0	0	0
	300 " α -estradiol	Kober	68	28	4	0	0	0	0	0

* The results with 75 ml of benzene as developing fluid and with the first 25 ml portion of benzene-methanol (9 1) were negative throughout

TABLE III

Recovery of Pure Estrogens from Column of Activated Alumina

Experiment No	Amount of estrogen added (a)	Color reagent used	Total estrogen found (b)		Recovery of added estrogen (b) (a) per cent
			9 1 eluate	4 1 eluate	
			γ	γ	
1	200 γ estrone	Kober	200	0	100
2	200 " "	"	195	0	98
3	100 " "	"	100	0	100
4	300 " α -estradiol	"	295	0	98
5	200 " estriol	"	0	160	80
6	300 " "	"	0	242	81
7	50 " estrone	Zimmermann	50	0	100
	50 " estriol	David	0	41	82
8	200 " estrone	Zimmermann	180	0	90
	200 " estriol	David	0	166	83
9	200 " estrone	Zimmermann	182	0	91
	200 " estriol	David	0	165	82
10	100 " estrone	Zimmermann	100	0	100
	25 " estriol	David	0	25	100
11	300 " α -estradiol	Kober	280		93
	200 " estriol	David	0	173	86
12	100 " α -estradiol	Kober	90		90
	100 " estriol	David	0	80	80

of estriol appears in the 9:1 eluate. This same effectiveness in partitioning binary mixtures is likewise demonstrated. As to over-all recovery of added estrogens, estrone and α -estradiol are recoverable to the extent of 90 to 100 per cent and estriol is consistently recovered to the extent of 80 to 85 per cent. Further elution with 4:1 benzene-methanol mixture reveals no detectable amount of estriol elution. An additional elution with a much stronger eluent, *viz.* pure absolute ethanol, does elute an additional 10 per cent of added estriol. However, the recovery of 80 to 85 per cent is very consistent except at very low concentration (25 γ) and therefore should introduce no serious handicap to the usefulness of the procedure.

DISCUSSION

In order to form a basis for the possible employment of the chromatographic technique for further purification of urinary estrogen residues, it becomes necessary to deal with amounts of estrogen most likely to be present, that is, of the order of 25 to 300 γ . The detection and estimation of this amount of active estrogenic material might be accomplished either by biologic assays or by colorimetric methods. In the absence of inactive contaminants, the colorimetric method seemed to be the method of choice.

The Kober reagent as modified by Venning *et al.* (11) produces a highly specific pink color with all three estrogens and because of its sensitivity and reproducibility in our hands has been employed for the percentage distribution studies involving single estrogens. For those studies involving mixtures of the estrogens, the David test which is specific for estriol among the three estrogens employed and the Zimmermann test which is specific for estrone among the estrogens employed were also used. Liquid chromatograms of various binary mixtures of the three naturally occurring estrogens demonstrated (Table II) that the percentage distribution was essentially unaffected by the presence of the second estrogen.

The recovery values listed in Table III are strong evidence that the naturally occurring estrogens can be quantitatively recovered from a chromatographic column of activated aluminum oxide and simultaneously separated into strongly phenolic (estriol) and weakly phenolic (estrone and α -estradiol) fractions. The anhydrous aluminum oxide is sufficiently inactivated under the conditions employed to render subsequent use of the column inadvisable.

SUMMARY

A study has been made of a number of suitable eluents for the crystalline estrogens, estrone, estriol, and α -estradiol, after adsorption on a column of activated alumina. The distribution of the three estrogens, both singly and in mixtures, in a series of solvents having increasingly powerful eluent

actions was investigated. Data have been presented to demonstrate the utility of the liquid chromatogram in the quantitative separation of the strongly phenolic estrogen (estriol) from the weakly phenolic estrogens (estrone and α -estradiol)

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LETTERS TO THE EDITORS

THE SCHARDINGER ENZYME IN BIOLOGICAL IODINATIONS*

Sirs

The ease of oxidation of iodide ion to free iodine by peroxides, especially rapid in the presence of a peroxidase, and the fact that hydrogen peroxide may be produced in living cells by many flavoprotein systems should make some biological systems containing such flavoproteins with their substrates possible sites of biological iodination

Milk, as is well known, contains the flavoprotein system xanthine oxidase which produces peroxide during the dehydrogenation of purines, and also contains a peroxidase and the readily iodizable protein, casein

Accordingly, experiments were carried out with unpasteurized milk. To the milk, from which the cream was poured off, were added small amounts of radioactive iodide ion, I^{131} , and a phosphate buffer. In various experiments, xanthine, catalase, and thiourea were added as indicated in the table

After standing for the specified times at 38° , the mixtures were made up to 2 N with respect to NaOH and hydrolyzed for 4 hours at 100° . The alkaline hydrolysate was extracted with butyl alcohol, and the alcohol layer washed with 5 N NaOH. The percentage of the total radioactive iodine remaining in the butyl alcohol layer is reported under Fraction A. The aqueous layer was acidified with H_2SO_4 and KI and excess KIO_3 added. The iodine was extracted with CCl_4 . Excess KI followed by excess KIO_3 was added and the mixture again extracted with CCl_4 . The iodine was then extracted from the CCl_4 with aqueous sodium thiosulfate. The percentage of the total iodine (radioactive) found in this fraction is reported under inorganic iodine. The residual radioactivity in the aqueous layer after the inorganic iodine and Fraction A were removed is reported under Fraction B. Fractions A and B of course represent organically bound iodine. All of the radioactivity measurements were carried out in solution in a glass cell surrounding the Geiger counter. Several other series of experiments, including one series in which hypoxanthine was employed as the substrate, gave results similar to those reported in the table.

The data show that the addition of substrates for the Schardinger enzyme to milk, in the presence of radioactive iodide ion, induces the formation of

* This investigation was aided by a grant from the Josiah Macy, Jr., Foundation

organically bound radioactive iodine Part of the organically bound iodine, like thyroxine, is readily extractable from the alkaline hydrolysate by butyl alcohol The iodination is inhibited by the addition of thiourea Thiourea, it has also been shown, inhibits the iodination of thyroid gland¹ The iodination proceeds in the presence of substantial amounts of a lyophilized horse liver catalase (obtained through the courtesy of Kurt Stern)

The failure of catalase to inhibit the reaction is not entirely clear The explanation may be that the peroxidase present effectively competed with

3 cc of 0.2 M phosphate buffer, pH 6.65 1 cc of radioactive I^{131} containing 0.5 μ of I^- , 38° for 45 minutes 5 cc of milk Total volume, 9 cc

Description	Per cent total I^{131} found in		
	Fraction A	Fraction B	Inorganic iodine fraction
Control	0.6	9	90
	0.6	9	90
0.45 mg xanthine	15	42	43
	16	43	41
0.45 " " 3 mg catalase	14	35	51
preparation	12	38	50
0.45 mg xanthine, 5 mg thiourea	0.5	8	91
	0.5	9	90

the catalase for the small amount of peroxide Furthermore, Keilin and Hartree have shown that the xanthine oxidase system is capable of performing oxidations even in the presence of catalase²

The formation of organically bound iodine by the xanthine oxidase system suggests an important rôle for the yellow enzymes in biological iodinations

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¹ Keston, A. S., Goldsmith, E. D., Gordon, A. S., and Charipper, H. A., *J. Biol. Chem.*, **152**, 241 (1944)

² Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **119**, 114 (1936)

CONCERNING THE MODE OF ACTION OF LIPOXIDASE

Sirs

It has recently been suggested by Hummel and Mattill¹ that lipoxidase is an *aerobic dehydrogenase*. Their suggestion was based on the behavior of the enzyme with certain inhibitors and the observation that 2,6-dichlorophenol indophenol was reduced more rapidly by crude enzyme plus lard ethyl esters than by enzyme alone. Previous work in our laboratory had led us to believe that lipoxidase could not function as a dehydrogenase, since methylene blue was not reduced anaerobically by lipoxidase plus cottonseed oil.

To confirm our earlier observation and to test the hypothesis of Hummel and Mattill further, we carried out experiments with crude soy bean extract and purified lipoxidase.

A solution of 2,6-dichlorophenol indophenol treated anaerobically with an extract of defatted soy bean meal (40 units of lipoxidase²) was decolorized in less than 2 hours. The reaction was not enhanced by ethyl linoleate or lard ethyl esters.¹ This extract, heated 15 minutes in a boiling water bath to destroy lipoxidase activity, bleached the dye anaerobically in 5 hours, with or without fat. No regeneration of lipoxidase activity occurred during this time. A purified lipoxidase preparation (1.5 mg of protein = 57 units of lipoxidase) failed to bleach the 2,6-dichlorophenol indophenol even after 48 hours incubation. (At this time, 80 per cent of the original enzyme activity was still present.)

Modified Thunberg tubes with hollow stoppers were evacuated by water pump 60 seconds after bubbling had subsided. 2 ml of substrate had been placed in the tube, 0.4 ml of enzyme solution in the hollow stopper, and these were mixed after the tubes were evacuated. The substrate consisted of 50 ml of H₂O, 0.6 mg of 2,6-dichlorophenol indophenol, 0.26 gm of sodium acetate, 2.5 ml of $\frac{2}{3}$ M phosphate buffer (pH 6.5), and 5 ml of acetone, or acetone containing 10 mg of fat, as indicated. All experiments were in duplicate.

We believe that the anaerobic reduction of 2,6-dichlorophenol indophenol by crude soy bean extract is independent of its lipoxidase activity because (1) the reaction does not require unsaturated fat, (2) destruction of lipoxidase in the crude extract did not completely destroy its ability to reduce 2,6-dichlorophenol indophenol, (3) a purified lipoxidase preparation comparable in activity to the crude extract failed to bleach the dye even after 48 hours incubation.

¹ Hummel, J. P., and Mattill, H. A., *Proc Soc Exp Biol and Med*, 55, 31 (1944).

² Balls, A. K., Axelrod, B., and Kies, M. W., *J Biol Chem*, 149, 491 (1943).

Kühne remarked that he would find a place for me in the laboratory at once." Thus began the relationship to Kühne as student, assistant, coworker, and friend, which continued until the former's death more than twenty years later. On Chittenden's return to Yale, he undertook with his students a research project on the primary cleavage products of proteins by digestive enzymes in close cooperation with the Heidelberg group, a project which continued for eight years. The interest of the Yale group was thus focused early on proteins, on proteolytic enzymes, and on the physiology of the gastrointestinal tract, an interest which continued for many years. Chittenden received the degree of Doctor of Philosophy in 1880, and in 1882 was appointed Professor of Physiological Chemistry in the Sheffield Scientific School, a position which he continued to hold until he became an emeritus professor in 1922.

The new laboratory soon attracted students, and by 1885 graduate work in physiological chemistry was well under way, a total of eleven papers by Chittenden and his students appearing during the college year of 1884-85. From the modest beginning of 1874, the laboratory of physiological chemistry of the Sheffield Scientific School developed, a laboratory which, under the leadership of Chittenden and his pupil and colleague, Lafayette B. Mendel, pioneered in the training of teachers and investigators in physiological chemistry and chemical physiology.

Chittenden's demonstrated ability in organization and administration resulted in his selection as administrative head (director) of the Sheffield Scientific School in 1898, a position which he occupied until his retirement in 1922. Although the increased burden of administrative duties made it necessary for him to relinquish much of his active teaching and research, his interest was still centered in physiological chemistry, and he continued to meet one class in the physiology of nutrition until 1916.

Chittenden's organization of the work in physiological chemistry was not confined to the Yale group. In 1898 he was requested by Columbia University to organize a Department of Physiological Chemistry at its College of Physicians and Surgeons. For five years, he devoted one day a week to this work, and, at the end of this period, the department was placed in charge of his pupil, William J. Gies.

During the period in which the organization of the Yale laboratory was his chief concern, the horizons of the biological sciences related to medicine were being extended. The American Physiological Society was organized on December 30, 1887, present at the organization meeting, and a charter member, was Chittenden, who in 1890 was elected a member of the Council, a position in which he served for fifteen consecutive years. In 1896, he was elected president of the Society and was reelected in successive years until 1904, a period of nine terms, the longest service in the history of the

Society In the meantime, biological chemistry had come of age as a separate discipline, so that it seemed wise to organize a national biochemical society At the organization meeting held in New York on December 26, 1906, Chittenden was among those present and as the leading biochemist of the country was selected as the first president of the American Society of Biological Chemists When in 1933 developments in the field of nutrition were such that a new organized group, the American Institute of Nutrition, was warranted, Chittenden was again included in the list of charter members

His interest in the various professional societies continued long after his retirement from active university life His last participation in the meetings of the Federation of American Societies for Experimental Biology was in 1938 at Baltimore, when the semicentennial of the American Physiological Society was celebrated Here he was an honored guest and spoke briefly at the banquet of the American Institute of Nutrition and was one of four of the charter members of the Physiological Society who were assembled for the semicentennial celebration

Chittenden was active in the editorial work of various journals With Bowditch of Boston and Howell of Baltimore, he represented physiology in the group of editors of the *Journal of Experimental Medicine* (1896), he was one of the seven members of the Physiological Society who comprised the first editorial committee of the *American Journal of Physiology* (1898)

In the critical period at the beginning of the present century, when the enforcement of the new food and drug legislation was beset with difficulties, the services of Chittenden were placed at the disposal of the United States Department of Agriculture He was a member of the Referee Board of Consulting Scientific Experts (commonly known as the Remsen Board) and not only served as a consultant but also in his own laboratory personally supervised studies on the influence of various food preservatives on the nutrition and health of man His experiments with sodium benzoate (published as a part of the report of the Referee Board in 1909) serve as a model of carefully planned, well conducted, and conservatively interpreted research in this field

Of the researches conducted under Chittenden's direction, his experiments on the protein element in human nutrition are of particular interest They were carried out in a period in which the dietary standards of Carl Voit were accepted without serious question These standard diets included relatively large amounts of protein (118 gm daily) In extensive experiments on subjects which included himself and his colleagues, Mendel and Underhill, typical Yale students, and a group of enlisted men assigned to New Haven by the Surgeon General of the United

States Army, Chittenden was able to prove that good health, physical vigor, and nitrogen balance could be maintained on levels of protein intake far below those of the Voit standard diet. These results, although highly controversial at the time of their publication, are now accepted with little question.

It is not the purpose of this memorial to present a detailed account of the many honors conferred in recognition of the achievements of this pioneer worker in biological chemistry. He had seen the development of physiological chemistry and nutrition in this country for more than sixty years. His book, "The development of physiological chemistry in the United States" (1930), presents these developments as he saw them. An unpublished manuscript, the work of his later years, awaits publication. It is concerned with the history of the American Society of Biological Chemists, in whose organization he participated and of which he was the first president.

HOWARD B. LEWIS

ACID CARDIOLIPIN AND AN IMPROVED METHOD FOR THE PREPARATION OF CARDIOLIPIN FROM BEEF HEART

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In a previous report (1) the isolation of a new phospholipid from beef heart was described. The name "cardiolipin" was suggested for this substance, which is of particular interest in connection with serodiagnostic tests for syphilis (2). The original method of preparation was tedious and involved considerable losses. An improved and simplified method is here described. The development of the new method depended in part on a study of the properties of the acid form of cardiolipin, the preparation of which is now reported for the first time.

The results of a study of the cleavage products of cardiolipin will be reported later. It should be noted at this time, however, that the original report of the presence of a carbohydrate component in the molecule was in error. The results obtained were apparently due to persistent traces of carbohydrate impurities.

EXPERIMENTAL

Preparation and Properties of Acid Cardiolipin

It was previously noted (1) that attempts to prepare the acid form of cardiolipin by the usual methods were unsuccessful. The difficulty appeared to be due to partial hydrolysis of the acid during the course of preparation. Attempts to remove excess mineral acid by dialysis of acidified mixtures or by washing ether or petroleum ether solutions with water resulted in gradual loss of phosphorus. The method finally employed depends for success on its rapidity and on the fact that the material is only very briefly exposed to water. The sodium chloride formed in the reaction is removed by a single partition between water and petroleum ether, and excess hydrochloric acid is then removed by repeated evaporation *in vacuo* with successive additions of alcohol.

To a solution containing 1 gm. of sodium cardiolipin in about 100 ml. of alcohol were added 10 ml. of N HCl, and the mixture was rapidly concentrated *in vacuo* to about 20 ml. and diluted with 40 ml. of water. The suspension was extracted twice with petroleum ether, no emulsions formed, and the layers separated quickly. The petroleum ether solution was evaporated to dryness *in vacuo*, the residue was dissolved in 20 ml. of absolute alcohol, and the alcoholic solution was rapidly concentrated to dryness.

in vacuo The addition of alcohol and subsequent evaporation to dryness were repeated five times Throughout this procedure air was excluded by carbon dioxide

The acid so prepared was a viscous yellowish oil weighing 0.9 gm, readily soluble in acetone or alcohol as well as in the other common lipid solvents It was not soluble in water In absolute ethyl alcohol it had $[\alpha]_D^{25} = +5.8^\circ$ The iodine number was 119 and the apparent molecular weight by titration was 726 It contained 4.31 per cent phosphorus, no sodium or chlorine was detected An alcoholic solution of the acid gave only traces of gummy precipitates with cadmium chloride, barium chloride, or calcium chloride

A sample of the acid which had been neutralized with potassium hydroxide on the same day that it was prepared was indistinguishable in serologic activity from the original sodium salt However, another sample of the acid which had been dissolved in absolute alcohol and kept in the refrigerator 3 days before being tested was found to have suffered a slight but significant loss in serologic activity The change was irreversible the activity was not restored by neutralization This observation indicated that the instability of the acid was not merely a matter of susceptibility to hydrolysis

A solution of the acid in absolute alcohol was stored at $3-6^\circ$, protected from light At intervals the optical rotation was redetermined, at the end of 3 weeks it had dropped to $+3.3^\circ$ No further change in rotation was detected after another week of storage in the refrigerator At this time the complement-fixing activity was very weak The acidity of the solution, determined by titration with alcoholic KOH, was unchanged, as was also the iodine number The solution, still protected from light, was then transferred to room temperature, about 25° There was a further fall in optical activity, after 4 weeks at room temperature the specific rotation was less than $+0.6^\circ$ At this time no serologic activity could be detected The acidity of the solution had increased slightly, the apparent molecular weight by titration was 650

It was noted above that when acid cardiolipin was rapidly prepared and then immediately neutralized the regenerated salt, obtained within 4 or 5 hours after the beginning of the experiment, had not undergone any detectable change as compared with the original material It is therefore possible, with proper precautions, to make practical use of the solubility of the acid in acetone in order to improve one of the steps in the preparation of cardiolipin, as described below

New Method for Preparation of Cardiolipin

Three major changes have been introduced (1) Methyl alcohol is substituted for ethyl alcohol in the extraction of the lipids from the tissue,

because it is a better solvent for cardiolipin than is ethyl alcohol and because it is also a good solvent for BaCl_2 . (2) The active fraction is precipitated from the crude extract with BaCl_2 instead of CdCl_2 . This eliminates the necessity for further fractionation to remove lecithin, which remains in the filtrate from the BaCl_2 precipitation. Similar results have been obtained by precipitation with CaCl_2 but the yields were not quite so good as those with the BaCl_2 method. (3) Cardiolipin is separated from the alcohol-insoluble "cephalin" fraction by converting the mixture of phospholipid salts to the corresponding acids and fractionating the mixed acids with acetone. This is a much more rapid and efficient method than the original one, which depended on precipitating the cephalin from methyl alcohol solution with NaCl . (1)

The entire preparation has also been greatly simplified by the finding that the various salts of cardiolipin may be quantitatively converted to the sodium salt by simply shaking their ether solutions with a strong solution of sodium chloride.

Extraction—The preparation of the tissue and preliminary extraction with acetone are carried out as previously described (1). After the second acetone extraction the tissue is dried before a fan and ground to a fine powder. Comparison of results obtained with and without drying of the tissue has indicated that this step does not cause appreciable oxidation of the lipids, provided it is carried through rapidly and the tissue powder reextracted immediately.

The dried tissue powder is treated with 2 liters of methyl alcohol (U.S.P., 95 per cent) for each 300 gm and allowed to stand 5 days at room temperature with frequent shaking. At least two such extractions should be made, and the yield can be somewhat increased by a third and even a fourth extraction. In one preparation, when the tissue was extracted four times, it was found that about 75 per cent of the total yield of cardiolipin was obtained from the first two extracts.

Precipitation of Active Fraction—To the methyl alcoholic extracts a 20 per cent aqueous solution of BaCl_2 is added until no further precipitate forms and the mixture is then allowed to stand overnight or longer at 3–6°. The precipitate is collected by centrifugation and washed at least twice with methyl alcohol, then once or twice with acetone. The washed precipitate is dissolved in ether and the ether solution is vigorously shaken for 5 minutes with about one-fourth its volume of one-half saturated NaCl . Emulsions form but are readily broken by the addition of alcohol. The aqueous layer is separated and discarded and the ethereal solution shaken twice more with NaCl in the same manner, then dried on anhydrous sodium sulfate.

Preliminary Purification—The ethereal solution is filtered, concentrated to small volume by distillation, poured into about 10 volumes of absolute

alcohol, and the precipitate once more dissolved in ether and precipitated by pouring the ether solution into absolute alcohol. The precipitate is then treated with methyl alcohol (c.p., absolute). The methyl alcohol insoluble portion is dissolved in ether and the solution poured into about 8 volumes of methyl alcohol. The resulting precipitate is separated and the supernatant added to the first methyl alcohol solution. There is no appreciable loss of cardiolipin into the fraction insoluble in methyl alcohol, which consists largely of barium salts *not decomposed by the NaCl treatment*.

This procedure yields two fractions which contain cardiolipin. Fraction Et, soluble in ethyl alcohol, and Fraction Me, soluble in methyl alcohol. On account of the difference in the character of the accompanying impurities in these two solutions, they are fractionated separately.

Fraction Et—The ether is removed by distillation *in vacuo* and the solution is further concentrated if necessary until the solid content is at least 5 mg per ml. If any precipitate separates during the concentration, it is added to Fraction Me.

To the ethyl alcohol solution is added one-fifth its volume of distilled water and the dilute alcoholic solution is then treated with about 1 ml of 20 per cent aqueous BaCl_2 per 100 ml. The mixture is allowed to stand at 3–6° overnight. The precipitate is collected by centrifugation and washed once with methyl alcohol and once with acetone. The precipitate first separates as a gum, but on washing with the anhydrous solvents it is readily reduced to a solid powder, which is then “dissolved” in a minimal amount of ether.

The ether solution obtained at this point should be a stiff gel. The formation of a gel in ether is characteristic of the pure barium salt of cardiolipin, and the appearance of the ether solutions in early stages of purification is therefore a valuable guide to the nature of the mixture in hand, gel formation indicates that the mixed barium salts contain a large proportion of cardiolipin. When such gels are encountered, no attempt should be made to dissolve them by further dilution with ether. The concentrated ether gel is simply homogenized as completely as possible and an equal volume of acetone is added in small portions with stirring. This flocculates the barium salt, while colored impurities remain in the ether-acetone supernatant. The process is repeated until the supernatant is colorless or nearly so. The precipitate is then dissolved in a little chloroform to which a few drops of water are added, when fairly pure, the barium salt does not dissolve readily in dry chloroform. To the solution in wet chloroform, 5 volumes of 95 per cent alcohol are added and the mixture is chilled in ice. If the precipitated barium salt tends to be colloidal, it may be flocculated by the addition of a few drops of one-half saturated NaCl and vigorous shaking. The barium salt so purified is dissolved in wet ether and recon-

verted to the sodium salt by shaking with one-half saturated NaCl as before

Fraction Me—Attempts to fractionate various salts of this mixture have been unsuccessful. In the acid form, however, the mixture is readily separated, since the most troublesome impurities remain insoluble in acetone after treatment with acids. If the separation is carried out rapidly, no chemical or serologic difference can be detected between the portion of cardiolipin prepared by acid treatment and that prepared from Fraction Et with no reagents other than neutral salts.

Fraction Me is acidified by the procedure described above for the preparation of acid cardiolipin. The residue obtained after evaporation with alcohol is immediately treated with from 10 to 15 ml of acetone per gm and the mixture warmed in a water bath at about 50°, when a portion of the residue readily dissolves. The mixture is cooled in ice, the supernatant rapidly decanted, and the insoluble portion reextracted several times with acetone in the same manner. The acetone solution, containing acid cardiolipin, together with some impurities, is concentrated to a small volume *in vacuo* and diluted with about 10 volumes of 95 per cent alcohol, and the solution is neutralized to phenolphthalein with saturated aqueous barium hydroxide. All steps of the process from the original acidification of Fraction Me through the neutralization with Ba(OH)₂ must be completed on the same day, so that the time during which the cardiolipin fraction is kept in the acid form is held to a minimum. The precipitated barium salt, collected after refrigeration overnight, is purified in exactly the same manner as that obtained from Fraction Et, and the two lots of sodium cardiolipin, from Fraction Et and Fraction Me, are combined and dissolved in ethyl alcohol.

Final Purification—Although the material recovered from the barium salts is very nearly pure cardiolipin, it still retains small amounts of colored impurities and may also contain a trace of nitrogen. For final purification the ethyl alcoholic solution is precipitated with CdCl₂ and the Cd salt is reprecipitated once from benzene by ethyl acetate as described in the original method (1). This procedure gives a purer product than can be obtained by repeated reprecipitations of the original barium salt. The purified Cd salt is finally reconverted to the sodium salt by shaking its ether solution with one-half saturated NaCl, as described for the barium salt.

The yield of sodium cardiolipin from beef heart by this method is about 3 gm per kilo of dry tissue powder, or 0.6 gm per kilo of moist tissue, as compared with 0.4 gm obtained by the original method (1). For storage the compound should be dissolved in ethyl or methyl alcohol and kept in a refrigerator, protected from light.

DISCUSSION

From the observed instability of acid cardiolipin and its failure to yield solid precipitates with CdCl_2 , CaCl_2 , or BaCl_2 , it may be concluded that the compound as it occurs in crude beef heart extracts is in the form of a salt rather than the free acid. The serologic activity of such extracts is stable for long periods of time, and the cardiolipin present in them is readily precipitable by the salts named. The composition of the cardiolipin salt originally present is not yet known, but from the observed solubility in alcohol it seems probable that it is either a sodium or a potassium salt. This question obviously cannot be settled until some method is available for isolating the substance without recourse to precipitation by metallic salts. As was mentioned above, there appears to be no difference in serologic activity between the sodium and potassium salts of cardiolipin.

The nature of the spontaneous alteration of the acid cardiolipin molecule is not known but the increase in acidity and the complete loss of serologic activity suggest that it is not a simple racemization. If only one of the optical antipodes is biologically active, as is so often the case, one might expect that the *dl* form would retain half the serologic activity of the original substance.

In contrast to the instability of the acid, it may be mentioned that samples of the pure sodium salt have been kept for periods up to 18 months with no evidence of deterioration.

SUMMARY

An improved method for the preparation of cardiolipin is described, depending on precipitation by BaCl_2 from methyl alcohol extracts of tissue and purification successively over the barium and cadmium salts.

The preparation and properties of acid cardiolipin are described.

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THE α -GLOBULIN FRACTION OF THE SERUM OF NORMAL AND HYPOPHYSECTOMIZED RATS*

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Previous experiments (1-3) have shown that following hypophysectomy the serum globulin concentration of dogs and rats increases and at the same time the serum albumin level falls. In the above studies serum albumins and globulins were separated by salt fractionation (4). With the aim of checking our earlier results and of obtaining more detailed information regarding the changes in serum protein after hypophysectomy, we have made electrophoretic as well as chemical analyses of the sera of intact and hypophysectomized rats. In agreement with the observations of Jameson and Alvarez-Tostado (5) we find but two electrophoretic globulin fractions (usually designated as β - and γ -globulin) in the serum of normal rats. The third globulin component, usually designated as α -globulin and found in the sera of most species ((6-9), and others), is absent or, if present, occurs in a very low concentration. After hypophysectomy, however, the α -globulin fraction appears along with the two other globulin components and is in part responsible for the postoperative increase in serum globulin level. Some of the quantitative electrophoretic and chemical data obtained from the sera of intact and hypophysectomized rats are reported in the present communication.

EXPERIMENTAL

Male rats of the Long-Evans strain, maintained on a diet adequate for normal growth and reproduction (3), were used. Hypophysectomy was performed when the animals were 3 to 4 months old. 3 weeks post-operatively the hypophysectomized animals as well as the normal controls were anesthetized with intraperitoneal sodium amytal, the thorax opened, and the animals bled by direct cardiac puncture. After the blood had clotted, the serum was collected and used for the analytical studies.

Electrophoretic analyses were made in a Tiselius apparatus having a tall, single sectioned micro cell of 2 ml capacity (10). Each serum sample was diluted with 3 volumes of 0.02 M sodium phosphate buffer (pH 7.4) con-

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taining 0.15 M NaCl and was then dialyzed against 1 liter of the same buffer, which was also used to fill the electrode vessels of the apparatus.

Chemical fractionation of the serum proteins was carried out by the method of Campbell and Hanna (4) which depends on precipitation of the globulins by 20 per cent sodium sulfite. The total nitrogen of the resulting filtrate and of diluted serum was determined by the micro-Kjeldahl procedure. After correction for non-protein nitrogen, the nitrogen values were converted to protein by being multiplied by 6.25. Globulin concentration was calculated as the difference between total protein and albumin concentrations.

Results

The data obtained by the electrophoretic and chemical methods are given in Table I. The electrophoretic composition is given in arbitrary units derived from the pattern areas. The areas for γ -globulin also contain that due to the ϵ - or salt boundary.

As may be seen, both the electrophoretic and chemical data confirm previous reports (1-3) that after hypophysectomy the albumin level falls while that of total globulin rises, resulting in a considerable decrease in the ratio of albumin to globulin. It will be noted that the electrophoretic values for the albumin-globulin ratio are consistently higher than those obtained by chemical fractionation. This may be due to precipitation of a portion of the albumin along with the globulins by the salt fractionation.

The electrophoretic data (Table I and Fig. 1) clearly show the absence of the α -globulin fraction from the sera of the normal rats. In only three of the fourteen serum samples derived from normal rats was there any indication of α -globulin and in two of these three the concentration of this component was very low.

Following hypophysectomy, α -globulin was found (Table I and Fig. 2) in all but one of the sera examined. It is also of some interest that in these sera the γ -globulin concentration was also somewhat higher, whereas the level of β -globulin was lower than in the sera from normal rats.

It has been reported (11) that certain sera, when examined at alkaline pH, show two components (designated α_1 and α_2) between the albumin and β -globulin peaks. A few electrophoretic analyses were, therefore, made at higher pH values and in different buffer solutions from those used for the experiments discussed above. The buffers used as well as the pattern areas and mobilities are given in Table II. The type of pattern obtained in sodium diethylbarbiturate buffer at pH 8.5 is illustrated in Fig. 3. In the present studies, normal rat serum at pH 8.5 shows one well defined α globulin component and an indication of a second. Longworth has shown (11) that the component of higher mobility (α_1) is included with the albumin at lower pH values.

TABLE I

Electrophoretic and Salt Fractionation of Serum Proteins of Intact and Hypophysectomized Rats

Rat No	Age	Salt fractionation per cent				Electrophoretic fractionation*							Mobilities†		
		Total protein	Albumin	Globulin	Albumin/Globulin	Total protein	Albumin	Globulin			Albumin/Globulin	Albumin	Globulin		
								α	β	γ			α	β	γ
Intact rats															
	days														
‡	142-151	5 95	3 82	2 13	1 79	655	455	125	75	2 3	4 6	2 0	0 8		
§	125-127	5 80	4 00	1 80	2 22	675	485	115	75	2 6	4 6	2 2	1 0		
38	112	5 71	3 55	2 16	1 64	685	465	150	70	2 1	5 1	2 4	1 1		
39	112	6 21	3 90	2 31	1 69	640	480	95	65	3 0	4 7	2 1	1 1		
40	112	6 49	3 99	2 50	1 60	555	380	100	75	2 2	5 3	2 5	1 2		
41	112	6 23	4 00	2 23	1 80	590	405	115	70	2 2	4 9	2 3	1 0		
42	112	5 94	4 06	1 88	2 16	540	385	35	70	50	2 5	4 9	3 1	2 3	0 8
49	133	5 76	3 94	1 82	2 16	580	400	110	70	2 2	4 6	2 1	0 8		
50	133	5 72	3 74	1 98	1 89	590	390	115	85	2 0	4 6	2 0	0 9		
61	166					585	445	20	65	55	3 2	4 7	2 9	2 0	0 7
62	166					595	445	20	85	45	3 0	4 5	3 6	2 5	0 8
71	137	5 81	3 67	2 14	1 71	640	420	130	90	1 9	4 2	2 0	0 9		
72	137	5 86	3 45	2 42	1 42	610	370	130	110	1 5	4 3	1 9	0 7		
85	67					580	450	90	40	3 4	4 5	2 0	0 7		
Average		5 97	3 81	2 16	1 78	600	420	6 104	68	2 4	4 73	2 2	2 0	9	
Hypophysectomized rats															
	141-150	5 66	3 10	2 56	1 21	530	305	40	85	100	1 3	4 5	2 7	2 0	0 8
	125-127	5 66	2 90	2 76	1 05	565	330	50	70	115	1 4	4 7	3 3	2 3	1 2
48	133	6 19	3 40	2 79	1 22	680	450	40	70	120	1 9	4 5	3 1	2 1	0 9
51	133	5 60	3 16	2 44	1 29	455	255	90	110	1 3	4 7	2 3	1 2		
52	133	5 76	3 23	2 53	1 28	460	330	30	35	65	2 5	5 9	4 0	2 8	1 2
53	133	5 70	3 46	2 24	1 54	655	440	40	75	100	2 1	5 6	4 3	3 4	2 1
74	137	5 52	2 95	2 57	1 15	440	250	25	70	95	1 3	4 0	2 6	1 7	0 6
76	137	5 80	2 97	2 83	1 05	520	300	40	90	90	1 3	4 6	2 8	2 1	1 1
Average		5 76	3 20	2 56	1 25	535	337	29	72	97	1 7	4 9	3 4	2 4	1 2

The averages do not include values for the pooled samples

* Expressed in arbitrary units derived from descending pattern areas Buffer, 0.02 M sodium phosphate + 0.15 M NaCl, pH 7.4

† Mobilities, derived from descending patterns, are expressed as sq cm per sec and per volt $\times 10^5$

‡ Nine rats, pooled

§ Three rats, pooled

|| Eight rats, pooled

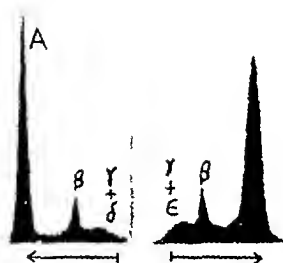


FIG 1

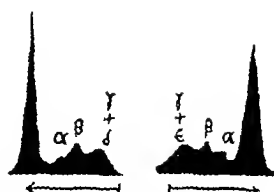


FIG 2

FIG 1 Typical electrophoresis pattern (Longworth scanning method) of normal rat serum. Left, ascending, right, descending pattern. Buffer, 0.02 M sodium phosphate + 0.15 M NaCl at pH 7.4. Field strength, 4.2 volts per cm. Time, 3 hours.

FIG 2 Typical electrophoresis pattern of serum from a rat 21 days after hypophysectomy. Left, ascending, right, descending pattern. Buffer, 0.02 M sodium phosphate + 0.15 M NaCl at pH 7.4.

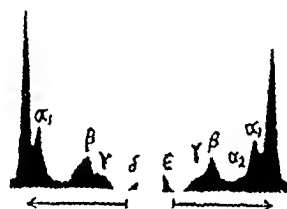


FIG 3 Electrophoresis pattern of normal rat serum. Left, ascending, right, descending pattern. Buffer, 0.1 N sodium diethylbarbiturate + 0.02 N barbituric acid at pH 8.5.

TABLE II
Electrophoretic Data at pH 8.0 and 8.5

Rat No	Type*	Buf fer†	pH	Areas of descending patterns arbitrary units						Mobilities from descending patterns $\times 10^4$ sq cm per volt per sec				
				Total pro- tein	Albu- min	Globulin				Albu- min	Globulin			
						α_1	α_2	β	γ		α_1	α_2	β	γ
71	N	P	8.0	455	320	25	10	70	30	6.3	5.0	4.4	2.9	1.8
42	"	V	8.5	540	310	110	25	80	15	5.9	5.1	4.7	2.9	1.8
40	"	"	8.5	625	350	130	15	100	30	6.0	5.1	4.2	2.6	1.5
8	H	"	8.5	645	250	100	60	150	85	5.7	4.9	4.2	2.8	1.9
pooled 76	"	"	8.5	810	330	180	75	150	75	5.8	4.9	4.7	2.8	1.6

* N = intact, H = hypophysectomized

† P = 0.02 M sodium phosphate, V = 0.1 N sodium diethylbarbiturate + 0.02 N barbituric acid

The reason for the presence or absence¹ of α -globulin in serum is not known. In humans its concentration is sometimes elevated in febrile diseases (8). The possibility, therefore, exists that the appearance of this globulin component after hypophysectomy is associated with undiagnosed infections.

A more likely possibility is suggested by the demonstration (1, 2) that thyroid insufficiency is associated with increased serum globulin which can be returned to normal by thyroid medication. It is thus probable that the thyroid insufficiency following removal of the pituitary gland is responsible for the appearance of the α -globulin in the serum. Experiments designed to test this possibility are in progress.

The authors wish to acknowledge the technical assistance of Miss Helen Sikorski and Miss Dorothy Wangerin.

SUMMARY

Sera from hypophysectomized and from intact, presumably normal, rats have been analyzed by electrophoretic and by salt fractionation methods. The ratios of serum albumin to globulin as determined by the two methods are quite similar and confirm the earlier finding that hypophysectomy results in a decreased albumin-globulin ratio.

Serum from normal rats quite consistently appears to differ from that of other species in that it lacks the electrophoretic α -globulin component but this component is present in the sera of hypophysectomized rats. Possible reasons for this difference are discussed.

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¹ Certain rabbit sera have been found to be deficient in α -globulin ((12) and unpublished data of Moore)

ADENYLPYROPHOSPHATASE AND MYOKINASE*

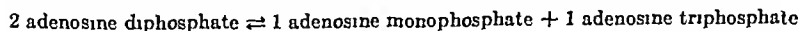
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Adenylpyrophosphatase is an enzyme which catalyzes the liberation of acid-labile phosphate from adenylyl pyrophosphate¹. The enzyme has been found in a large number of animal tissues, in yeast, and in certain plant tissues. Lohmann (1) found adenylpyrophosphatase in muscle, and Jacobsen (2) showed that the enzyme is specific and not identical with inorganic pyrophosphatase. A one-step dephosphorylation of adenosine triphosphate was first observed by Lohmann (3). He found that if the water-insoluble residue of lobster muscle is washed several times with water and then incubated with adenosine triphosphate only the third, terminal phosphate is liberated. This observation made it possible to isolate adenosine diphosphate. Engelhardt and Ljubimova (4) described recently the occurrence of adenylpyrophosphatase in the myosin fraction from rabbit muscle, and Ljubimova and Pevsner (5) found that myosin after a number of reprecipitations catalyzes the hydrolysis of only the terminal phosphate from adenosine triphosphate, leaving adenosine diphosphate as the end-product. Klemzeller (6) found that muscle extracts liberate only the terminal phosphate from inosine triphosphate. Colowick and Kalckar (7) observed a one-step transphosphorylation of hexose by adenosine triphosphate, catalyzed by yeast hexokinase.

In a previous article (8) a reaction, termed phosphate dismutation, has been described. This reaction, which is catalyzed by the acid-stable muscle enzyme, myokinase, is a reversible transfer of labile phosphate from 1 molecule of adenosine diphosphate to another according to the equation



Since myokinase regenerates adenosine triphosphate from diphosphate, addition of this enzyme to water-insoluble muscle proteins which split only one labile phosphate from the triphosphates should bring about the splitting of the second labile phosphate. The data to be presented in this paper

* This work was supported by a grant from the Rockefeller Foundation.

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¹ In this paper adenylyl pyrophosphate is used as a common term for adenosine tri- and diphosphate.

show that a rapid splitting of the second labile phosphate of adenosine triphosphate by a solution of myosin or a suspension of muscle residue actually can be accomplished if myokinase is added to such enzyme systems.

Liver extract and protein preparations from potato contain adenylypyrophosphatases which catalyze the dephosphorylation of adenosine diphosphate as well as triphosphate, although these systems are free of myokinase. However, the splitting of phosphate from the triphosphorylated compound is faster than from the diphosphorylated compound. Moreover, studies of these enzyme systems indicate that adenosine tri- and diphosphates, as well as inosine tri- and diphosphates, are split by a common enzyme which merely exhibits a greater specificity towards the triphosphorylated nucleoside. Hexokinase from bakers' yeast presents a strict specificity towards triphosphorylated nucleoside (7).

Methods

Phosphate determinations were carried out according to the method of Fiske and Subbarow (9). pH determinations were carried out with the Beckman glass electrode pH meter.

Adenosine tri- and diphosphates were separated according to the methods described in a previous paper (8).

Analysis of Adenosine Triphosphate—The ratio $P_{\text{total}}/N_{\text{total}}$ was 1.28, calculated, 1.33, $P_{\text{labile}}/P_{\text{total}}$ was 0.65, calculated, 0.67. Inosine triphosphate was prepared according to Lohmann (10). On analysis the product gave the ratio $P_{\text{total}}/N_{\text{total}}$, 1.58, calculated, 1.66, $P_{\text{labile}}/P_{\text{total}}$, 0.66, calculated, 0.67. When the inosine triphosphate was added to myosin, 50 per cent of the labile P was liberated as inorganic orthophosphate.

Adenylypyrophosphatase from Muscle Lohmann's Method (Cf (3))—Lobster muscle was minced and washed five times with 0.4 per cent KCl and finally suspended in veronal buffer at pH 7.6. About 1 ml of suspension was incubated with adenosine triphosphate.

Ljub.ova and Engelhardt's Method—Myosin was prepared according to Greenstein and Edsall (11). 50 gm of rabbit muscle were extracted with 0.5 M KCl containing 0.03 M bicarbonate and precipitated by dialysis against H_2O , or by dilution with 20 volumes of H_2O . The precipitate was washed twice with water, redissolved in 2.5 per cent KCl containing 0.05 per cent $NaHCO_3$, and again precipitated by dialysis or dilution. Bailly (12) has recommended Sørensen's glycine buffer (13), because it protects myosin against the action of heavy metals, glycine buffer was, therefore, used in a number of the experiments presented in this paper. In accordance with the findings of Bailly, a pH of about 9 was used and calcium chloride was added to the system.

An isoelectric precipitate was obtained from water extracts of muscle by

acidifying to pH 6. The precipitate formed was centrifuged, washed four to five times with water, and redissolved in 1 per cent KCl at pH 9.

Adenylpyrophosphatase from Liver—Liver extract was prepared by extracting minced liver with 2 volumes of water and subsequently dialyzing against 0.4 per cent KCl.

Adenylpyrophosphatase from Potato—Potato adenylpyrophosphatase has been obtained as a protein of very high catalytic activity. Berger, Colowick, and Slein³ recently found that water extracts of potatoes contain a highly active adenylpyrophosphatase which remains in solution after most of the other proteins have been precipitated by adding ammonium sulfate to about 60 per cent of saturation. The adenylpyrophosphatase in the supernatant fluid was precipitated by evaporating the solution until full saturation with ammonium sulfate was reached. The protein precipitate was collected on filter paper, redissolved in a small volume of water, and dialyzed against water in order to remove ammonium sulfate. The enzyme solution still contained considerable amounts of a dark brown pigment which was present in all the water extracts of potato.

Fractionation with ammonium sulfate yields an enzyme of high catalytic activity, 1 γ of protein per ml. splits about 5 to 6 γ of P from adenosine triphosphate containing 40 γ of terminal P in 5 minutes at 30° at pH 6.5, which is the pH optimum. Calcium, which is a necessary component of the system (*cf.* Table VII), was added in amounts of 0.5 mg. of CaCl_2 per ml. The test described here will be referred to as the standard test.

Aluminum hydroxide adsorbs a good deal of the pigment,³ leaving the enzyme in the supernatant fluid. 48 ml. of potato enzyme were acidified to pH 6 and 2.4 ml. of aluminum hydroxide 0.7% suspension (14) were added. The mixture was centrifuged and the precipitate was discarded. The total amount of protein in solution decreased from 175 mg. to 115 mg., the catalytic activity increased from 5 γ of P to 7 γ of P per microgram of protein in the standard test.

Fractionation with ethanol at pH 5.5 accomplished some purification, but with a considerable sacrifice of total activity. 50 ml. of the aluminum

Berger, L., Colowick, S. P., and Slein, M., unpublished observations.

³ If the difference between the amount of pigment before and after aluminum hydroxide adsorption is great, the comparison of the activities per mg. of protein (biuret) becomes less accurate. This is due to the fact that a small amount of the pigment remains in the supernatant fluid of the copper hydroxide, increasing the readings of the biuret color distinctly. Thus the increase in catalytic activity after adsorption on aluminum hydroxide (from 5 γ of P to 7 γ of P per mg. of protein per 5 minutes) may be smaller, owing to the difference in the quality of the biuret color. However, the loss in protein is correspondingly smaller. The alcohol fractionation does not give rise to any changes in the amount of pigment and the activity values are, therefore, always directly comparable.

hydroxide-treated enzyme solution were acidified to pH 5.5 with acetate buffer and cooled to 0°. 12.5 ml of ethanol were added and the mixture containing about 20 per cent alcohol was centrifuged at a low temperature. The precipitate which contains denatured proteins was discarded. An additional 12.5 ml of ethanol were added to the supernatant fluid, raising the alcohol concentration to 33 per cent. The precipitate obtained at this alcohol concentration contained the most active adenylypyrophosphatase. The precipitate was readily and completely dissolved in a small volume of water. The solution was dialyzed against 0.4 per cent KCl in order to remove traces of alcohol. The fraction contained about one-fifth of the protein of the original fraction, and split 9 γ of P per microgram of protein in the standard test.

Results

Muscle Adenosinetriphosphatase—The enzyme from skeletal muscle seems to be specific for adenosine triphosphate. The dephosphorylation of adenosine diphosphate involves the catalytic action of myokinase. This applies to myosin and lobster muscle suspensions as well as to the isoelectric precipitate from muscle extract (see Tables I to III).

The slight hydrolysis of adenosine diphosphate without added myokinase is presumably due to traces of myokinase present in this preparation. The rate of phosphate liberation in the presence of both adenosine tri- and diphosphate is lower than in the sample incubated with adenosine triphosphate alone (Table IV). The inhibitory effect of adenosine diphosphate is probably due to a competition with the active substrate, adenosine triphosphate, for the enzyme. In view of these findings there seems to be no need for assuming the existence of a specific adenosinediphosphatase (12).

Kleinzeller (6) reported recently that inosine triphosphate, obtained by chemical deamination of adenosine triphosphate, is dephosphorylated in the presence of myosin 3 to 5 times more rapidly than adenosine triphosphate. Ordinary muscle extract, however, was found to dephosphorylate the two nucleotides at about the same rate.

In the present studies, the adenosine triphosphate obtained in the usual way was subjected to a number of additional barium precipitations in 50 per cent alcohol and 0.1 N hydrochloric acid (6). These additional barium precipitations correspond to those employed when inosine triphosphate is freed from traces of nitrate and urea. It appears from Table V that the adenosine triphosphate is split at a somewhat slower rate than inosine triphosphate, but the difference in the rates of dephosphorylation is smaller than that reported by Kleinzeller (see Table V).

The significance of these observations will be discussed later.

TABLE I

Necessity of Myokinase for Dephosphorylation of Adenosine Diphosphate by Washed Lobster Muscle

20 gm of lobster muscle were washed five times with 0.5 per cent KCl and suspended in 200 ml of 0.03 M veronal buffer at pH 7.5. 2 ml of reaction mixture contained 1 ml of suspension, 1 mg of MgCl₂, 2 mg of glutathione, 20 γ of myokinase, and one of the following substrates: (a) adenosine triphosphate (ATP) (80 γ of labile P) or 200 γ of pentose, (b) adenosine diphosphate (ADP) (70 γ of labile P) or 350 γ of pentose. The mixtures were incubated for 10 minutes at 20° at pH 7.5.

	P	P split
	γ	γ
ATP, initial	24	
" incubated	51	26
" " with myokinase	84	60
ADP, initial	19	
" incubated	28	9
" " with myokinase	66	45

TABLE II

Necessity of Myokinase for Dephosphorylation of Adenosine Diphosphate by Myosin

Myosin (from rabbit) precipitated three times by dialysis against water and suspended in 1 per cent KCl containing 0.02 M sodium bicarbonate, 1 ml per sample. Magnesium chloride, 0.5 mg, myokinase (Fraction 2), 10 γ + 2 mg of glutathione (pH 8), ADP (50 γ of labile P), incubated for 10 minutes at 25°.

	P liberated
	γ
ADP	4.5
" + myokinase	25.0

TABLE III

Necessity of Myokinase for Dephosphorylation of Adenosine Diphosphate by Precipitate from Muscle Extract

Muscle extract (water extract) was adjusted to pH 6 and the precipitate formed was washed four times with water and suspended in 0.5 per cent KCl, 0.5 ml per sample. Substrates, ATP and ADP (corresponding to 80 γ of labile P), magnesium chloride, 0.5 mg, myokinase, 20 γ + 2 mg of glutathione (pH 8.0), incubated for 10 minutes at 30°.

	P split
	γ
ATP	33.0
ADP	2.3
" + myokinase	11.0

Liver Adenylpyrophosphatase—Liver extracts liberate phosphate from adenosine di- as well as triphosphate. Since liver tissue and extract are free of myokinase, the dephosphorylation of adenosine diphosphate must be attributed to a direct dephosphorylation. It seems most likely that the dephosphorylation of adenosine tri- and diphosphate is due to one and the same enzyme.

TABLE IV

Inhibition of Muscle Adenosinetriphosphatase by Adenosine Diphosphate

Myosin reprecipitated twice, 1.2 mg of protein per sample. Substrates, ATP (165 γ of labile P), pH 7.5, ADP (175 γ of labile P), pH 7.5, glycine buffer, 0.1 M, pH 9, CaCl₂, 0.5 mg, incubated for 10 minutes at 30°.

	P split
	γ
ATP	190
ADP	40
ATP + ADP	145

TABLE V

Dephosphorylation of Adenosine and Inosine Triphosphate by Myosin Solution

Myosin (from rabbit) reprecipitated twice, redissolved in 2.5 per cent NaCl. 2 ml of this solution (i.e. 1.5 mg of protein) were added to each sample. The pH was adjusted by adding 1 ml of 0.1 M glycine buffer, pH 8.8 or 9.4. CaCl₂, 0.5 mg, was added to all samples. The substrates, (ATP)* and inosine triphosphate (ITP), were incubated with the enzyme for 3 minutes at 30°.

Substrate	Labile P in substrate	pH of mixture	P liberated
	γ		γ
ATP	230	8.5	31.4
ITP	230	8.5	54.6
ATP	230	9.3	64.0
ITP	230	9.3	84.0
ATP	110	9.2	36.0
ITP	110	9.2	48.0

* The ATP was subjected to three additional precipitations with barium and alcohol in 0.1 M HCl in analogy with the reprecipitation of the ITP.

The inhibition of adenosinetriphosphatase by adenosine diphosphate has also been observed in liver extracts (Table VI). The triphosphate compound is split at a rate about twice that of the diphosphate. The rate of splitting in the presence of both substrates is very nearly the average of the rates with the two substrates individually.

Potato Adenylpyrophosphatase—The different methods of preparation of adenylpyrophosphatase from potato and from muscle suggest that the two

enzymes have different chemical properties. Both enzymes require the presence of calcium ions (12). In the absence of calcium ions, the activity of the potato enzyme is less than half of that found in the presence of calcium (see Table VII). Otherwise, the enzymatic properties of the two adenylypyrophosphatases differ considerably. Thus, whereas the muscle enzyme has a very alkaline pH optimum (12), the potato enzyme has a rather acid pH optimum, around pH 6.5. In Fig. 1 the enzyme activity (ordinate) is plotted against pH (abscissa).

TABLE VI

Inhibition of Liver Adenosinetriphosphatase by Adenosine Diphosphate

Enzyme, dialyzed liver extract, 0.2 ml per sample, substrates, ATP (110 γ of labile P), pH 7.97, ADP (140 γ of labile P), pH 7.97, buffer, 0.05 M borate (pH 7.82), MgCl_2 , 0.3 mg, incubated for 7 minutes at 28°. P_0 directly determined P, P_{10} after 10 minutes hydrolysis at 100°

	P_0	P_{10}	P split
	γ	γ	γ
ATP, initial sample	14.4		
ADP, " "	9.3		
ATP + ADP, initial sample	15.3		
" incubated sample	31.0	122.0	16.6
ADP, " "	18.7	152.0	9.4
ATP + ADP, incubated sample	29.0	282.0	13.7

TABLE VII

Stimulation of Potato Adenylypyrophosphatase by Calcium Ions

Enzyme, potato adenylypyrophosphatase (6 γ of protein), substrate, ATP (90 γ of labile P, 45 γ of terminal P), buffer, 0.1 M sodium succinate, pH 6.5, incubated for 5 minutes at 30°

	P split
	γ
Without CaCl_2	14.0
With CaCl_2 (0.4 mg)	31.5

The potato enzyme is an adenosinediphosphatase as well as a triphosphatase, although the triphosphorylated compound is split somewhat faster than the diphosphorylated when the compounds are added in equimolar amounts. The rate of splitting in the presence of both compounds is very nearly the average of the rates of splitting in the presence of tri- or diphosphate separately (see Table VIII). This inhibitory effect of adenosine diphosphate upon the dephosphorylation of triphosphate has already been mentioned in connection with muscle and liver adenylypyrophosphatase.

and has been interpreted as a competition effect. If adenosine diphosphate is added in twice the molar concentration of that of triphosphate (Table IX) the rates of splitting of the two nucleotides are very nearly the

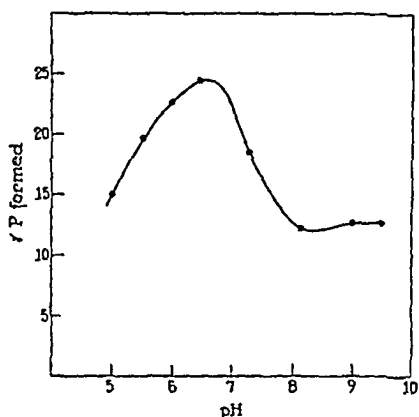


FIG 1 Activity of potato adenylypyrophosphatase at different hydrogen ion concentrations

TABLE VIII

Inhibition of Potato Adenosinetriphosphatase by Adenosine Diphosphate

Enzyme, potato adenylypyrophosphatase, second alcohol precipitate, 1 γ of protein per ml, substrates, ATP (80 γ of labile P), pH 6.50, ADP (40 γ of labile P), pH 6.52, both nucleotides (120 γ of labile P), buffer, 0.1 M sodium succinate, pH 6.5, 0.2 ml per sample, CaCl₂, 0.2 mg, volume, 1 ml, incubation, 2 and 4 minutes, temperature, 30°

	Time	Inorganic P	P split
	min	γ	γ
ATP	0	2.6	
"	2	13.2	10.6
"	4	23.0	20.4
ADP	0	1.4	
"	2	8.2	6.8
"	4	14.2	12.8
Both nucleotides	0	3.6	
" "	2	12.0	8.4
" "	4	20.4	16.8

same. Potato adenylypyrophosphatase liberates both labile phosphate groups in inosine triphosphate at about the same rate (see Fig 2). Unlike the myosin enzyme (6) the potato enzyme dephosphorylates the adenine nucleotide faster than the inosine nucleotide (Table IX).

Adsorption of Potato Adenylpyrophosphatase on Myosin—According to Engelhardt *et al* (15) myosin is identical with muscle adenosinetriphos-

TABLE IX

Dephosphorylation of Adenosine Tri- and Diphosphate and of Inosine Triphosphate by Potato Enzyme

Potato enzyme (40 γ of protein), CaCl_2 , 0.4 mg, 0.2 ml of 0.1 M succinate buffer, pH 6.33, ATP (120 γ of labile P), ADP (105 γ of labile P), ITP (130 γ of labile P), 30°

Substrate	Time	P	P split
	min	γ	γ
ATP	0	1.5	
ADP	0	5.7	
ITP	0	2.9	
ATP	5	68.0	66.5
ADP	5	67.5	61.8
ITP	5	40.5	37.6
ATP	15	116.0	114.5
ADP	15	98.3	98.3
ITP	15	93.0	90.1

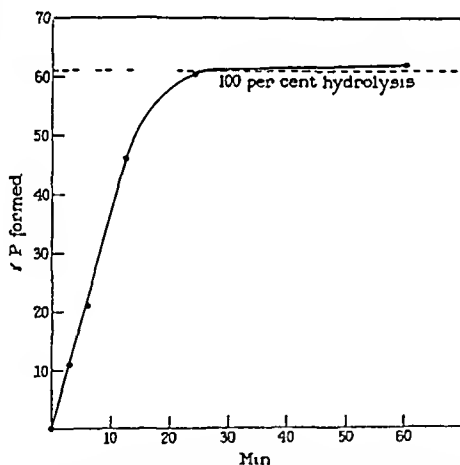


FIG 2 Liberation of phosphate from inosine triphosphate by potato adenyl pyrophosphatase. The broken line indicates the amount of phosphate which corresponds to complete hydrolysis of the labile phosphate in the inosine triphosphate

phatase. This view has been supported from various quarters (12, 16). The possibility that the presence of this particular enzyme in myosin is due to adsorption cannot be excluded, however. In this connection it may

TABLE X

Adsorption of Potato Adenylpyrophosphatase on Myosin

To 5 ml containing 1.5 to 2 mg of globulin (myosin or fibrinogen) was added 0.2 ml of potato enzyme (containing 50 γ of protein). The mixture was then diluted with H_2O up to 100 ml. The precipitated globulin was centrifuged and washed with 40 ml of water and then dissolved in 2 per cent KCl. ATP was added in excess. The pH was between 8.5 and 9.0.

Enzyme	P split per 5 min	Excess split	Adsorbed
	γ	γ	per cent
Myosin	27.2		
“ with adsorbed potato enzyme	74.8	47.6	37
“ and potato enzyme*	91.8	64.6	
“ (inactive)	6.7		
“ with adsorbed potato enzyme	152.0	145.3	35
“ and potato enzyme*	215.0	208.3	
Fibrinogen	2.4		
“ with adsorbed potato enzyme	8.5	6.1	2.5
“ and potato enzyme	120.0	117.6	
“	0.0		
“ with adsorbed potato enzyme	11.0	11.0	2.0
Potato enzyme	275.0	275.0	

* The amount of potato enzyme corresponds only to half the amount employed in the adsorption sample.

TABLE XI

Adsorption of Potato Adenylpyrophosphatase (Adenosinediphosphatase) on Myosin

To 20 ml of myosin solution (reprecipitated once) were added 1.5 ml of potato enzyme (crude ammonium sulfate precipitate), after a 5 minute incubation at 30° the mixture was diluted with chilled distilled water to 400 ml. The precipitate formed was further washed with 50 ml of water and then redissolved in 2.5 per cent NaCl. The final volume was 27 ml. Myosin without the addition of potato enzyme was subjected to the same treatment.

For the enzymatic test 2 ml of the myosin solutions containing 0.5 mg of $CaCl_2$ were mixed with 1 ml of buffer and 0.1 ml of substrate. The buffers were glycine + NaOH, pH 9.05, glycylglycine, pH 6.9, and sodium succinate, pH 6.5. The substrates were ATP (110 γ of labile P) and ADP (105 γ of labile P). The mixtures were incubated for 10 minutes at 30°.

	P formed
	γ
Myosin potato enzyme + ADP, incubated at pH 6.9	22.0
“ “ + “ “ “ 9.05	12.4
“ “ + ATP, “ “ “ 6.5	51.6
“ “ + “ “ “ 9.05	44.6
Myosin + ADP, incubated at pH 9.05	0.8
“ + ATP, “ “ “ 9.05	42.8

be of interest to present some experiments in which small amounts of a highly active potato adenylypyrophosphatase were added to myosin. It appears from Table X that even after 20-fold dilution of myosin and subsequent washing of the precipitate a considerable proportion (30 to 60 per cent) of the water-soluble potato enzyme remains in the myosin fraction which now splits both ADP and ATP at pH 6.5 as well as at 9 (Table XI). When fibrinogen and potato enzyme were mixed no adsorption was seen.

DISCUSSION

It has been the general belief (4, 12) that muscle contains two different adenylypyrophosphatases: adenosinetriphosphatase, which occurs particularly in myosin (4), and adenosinediphosphatase, which is found in the water-soluble part of muscle extracts. From what has been described in the present paper, it is, however, clear that the two-step dephosphorylation of adenosine triphosphate in muscle extracts may just as well be attributed to the joint action of adenosinetriphosphatase and myokinase. The latter enzyme converts the adenosine diphosphate, formed by the action of the former enzyme, into adenylic acid and adenosine triphosphate which is acted upon by the adenosinetriphosphatase, and so on. In the final balance, adenosine triphosphate will be dephosphorylated to adenylic acid.

When adenosine diphosphate is dephosphorylated and no myokinase is present, it is obvious that the reaction must be due to direct adenosinediphosphatase action. However, it does not follow from this that adenosinediphosphatase is an enzyme different from adenosinetriphosphatase. The latter enzyme may merely be less specific in liver than it is in muscle, so that it acts not only on adenosine triphosphate but also on the diphosphate. It has already been pointed out that the two nucleotides are probably dephosphorylated by one common enzyme and not by two different enzymes.

It is difficult at the present time to evaluate Kleinzeller's observation, that inosine triphosphate is dephosphorylated markedly faster than adenosine triphosphate when incubated with myosin. It can mean either that inosine triphosphate is the biological substrate of myosin triphosphatase or that the adenosine triphosphate used contained traces of an inhibitor which is inactivated by deamination.

Engelhardt has advanced the hypothesis that muscle adenosinetriphosphatase is identical with myosin. A number of other investigators (12, 16) have lent their support to Engelhardt's claim.

From the experiments presented in this paper and in particular from the observation of the adsorption of potato adenylypyrophosphatase on myosin fractions, it seems obvious that we are not yet in a position to distinguish between the two alternatives, whether myosin is identical with the triphos-

phatase, or whether the enzyme is adsorbed on the myosin molecule. The situation is analogous to that encountered in studies of the occurrence of phosphatase in viruses (17), in which case the alternatives are adsorption from the tissues on the one hand and actual incorporation in the virus on the other.

It may be emphasized that even an adsorption of triphosphatase on myosin might well connect pyrophosphate hydrolysis with the mechanical phenomenon in the muscle fiber.

SUMMARY

Adenylpyrophosphatases from various animal and plant tissues have been studied.

1 Adenylpyrophosphatase from myosin and from muscle extracts is a specific adenosinetriphosphatase. This was shown by separation of the water-soluble protein components from the active globulins. After such a separation only adenosine triphosphate but not the diphosphate is dephosphorylated. The diphosphonucleoside is hydrolyzed to monophosphonucleoside (adenylic acid) upon further addition of myokinase, which catalyzes the reversible reaction, $2 \text{ adenosine diphosphate} \rightleftharpoons 1 \text{ adenosine monophosphate (adenylic acid)} + 1 \text{ adenosine triphosphate}$, and thus transforms half of the adenosine diphosphate into triphosphate, which is subsequently hydrolyzed by the triphosphatase. The dephosphorylation of adenosine tri- as well as diphosphate in fresh muscle extracts is probably due to the combined action of adenosinetriphosphatase and myokinase. The existence of a separate adenosinediphosphatase has not been observed and is considered unlikely.

2 Liver extract hydrolyzes adenosine triphosphate to adenylic acid with the liberation of two phosphate groups. Since the extracts are free of myokinase, the adenylpyrophosphatase in liver is combined tri- and diphosphatase. The two reactions may be catalyzed by the same enzyme, which is merely more active toward adenosine tri- than it is toward adenosine diphosphate.

3 The albumin fraction of potato extracts contains a highly active adenylpyrophosphatase which hydrolyzes adenosine triphosphate to adenylic acid and two phosphate groups. Since myokinase is absent, the dephosphorylation proceeds directly. The first step proceeds faster than the second. The rate in the presence of both nucleotides is near the average of the rates of the two steps, thus indicating one common phosphatase for adenosine tri- and diphosphate.

Inosine triphosphate is hydrolyzed by the potato enzyme, the end products are inosine monophosphate (inosinic acid) and two phosphate groups.

The inosine nucleotides are dephosphorylated at about half the rate found with adenine nucleotides

The potato adenylpyrophosphatase is about 50 to 100 times more active per mg of protein than is the myosin enzyme. If a small amount of the potato enzyme is added to myosin and the latter is precipitated by 20-fold dilution with water and washed, a large proportion of the potato enzyme remains in the myosin fraction.

The corresponding procedure performed with fibrinogen and potato enzyme did not give rise to any appreciable adsorption of potato enzyme.

The adsorption of potato adenylpyrophosphatase on myosin raises the question as to whether the occurrence of so large a proportion of muscle adenosinetriphosphatase in the myosin fraction might not be attributed to an adsorption of this enzyme on myosin.

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THE METABOLISM OF TYRAMINE, *L*-TYROSINE, AND PHENOL BY RAT TISSUES IN VITRO

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Ewins and Laird (1) showed that tyramine disappeared when perfused through the heart, liver, or uterus of rabbits and cats. The amine oxidase, which deaminates tyramine and many other primary amines, has been studied in liver and kidney (2) but no further observations have been made on the fate of tyramine in the heart or other muscle. In particular it is not known whether muscle is able to deaminate the side chain of tyramine or break the nucleus or both. It was therefore of interest to investigate this question with the tissue slice technique. Tyramine, *L*-tyrosine, and phenol were chosen for comparison, and the changes in the amino nitrogen of the first two and the hydroxy groups of all three compounds were estimated after incubation with various tissue slices.

EXPERIMENTAL

The tissues of the rat were used. The kidney and liver slices were made in the usual way. The heart was prepared by cutting across the ventricles so that many thin rings of muscle were made, and these were washed free of blood. The skeletal muscle was taken from the hind legs and cut transversely to the long axis of the fibers. The biceps femoris muscle proved the most convenient to handle. The smooth muscle was taken from the ileum. It was split open, washed with saline, and the mucosa carefully removed by scraping. The muscle was then cut into strips. The tissues were placed in 50 cc flasks containing 40 cc of the Krebs-Henseleit (3) solution and shaken at 37° in an atmosphere of 5 per cent CO₂ and 95 per cent O₂. At the end of the experiment appropriate amounts of tyramine, *L*-tyrosine, or phenol were added to certain of the controls to serve as standards and 10 cc of 20 per cent trichloroacetic acid was then added to all the vessels. The precipitated protein was centrifuged down. The hydroxy groups were estimated by the diazotized *p*-nitroaniline reagent according to the method of Theis and Benedict (4). This reagent gives an orange-red color with monohydroxyphenyl compounds. Dihydroxyphenylalanine gives a brownish color which was not produced in our experiments and aliphatic hydroxy compounds give no color. The amino nitrogen was determined in the Van Slyke apparatus and the values given

below represent the difference between that obtained on the tissue alone and that obtained on tissue incubated with either tyrosine or tyramine. Tyramine gives off its nitrogen quantitatively after 5 minutes with nitrous acid in the Van Slyke apparatus.

The disappearance of hydroxyphenyl groups after incubation with tissues may be due to their conjugation with sulfuric or glycuronic acids or to the breaking of the ring. 1.0 cc of concentrated HCl was added to the protein-free trichloroacetic acid solution and the mixture boiled for 30 minutes. It has previously been shown (5) that boiling for 10 minutes with 0.25 cc of concentrated HCl will completely hydrolyze phenol sulfate. The hydrolyzed solutions were compared with untreated controls and no evidence was obtained that the disappearance of the hydroxy groups of tyramine or tyrosine was caused by conjugation. Phenol is known to be conjugated by liver slices (5), but not by other tissues. Thus after 4 hours incubation 0.28 mg of tyramine disappeared out of 0.5 mg added at the beginning to skeletal muscle. After the material was boiled with acid, the value obtained was 0.27 mg, showing that conjugation had not occurred. Out of 0.5 mg of phenol added to skeletal muscle 0.20 mg disappeared and after boiling the same value was obtained. For heart the respective values with tyramine were 0.22 and 0.19 mg, for liver 0.14 and 0.15 mg. It thus may be concluded that the disappearance of hydroxy groups from the added compounds in the tissues is not due to conjugation but is probably the result of the breaking of the ring. The possibility, however, remained that the apparent disappearance was not due to the destruction but rather to the adsorption of the compounds onto the proteins of the tissue when they are precipitated with trichloroacetic acid. That this is not the case is shown by the following experiments. Tyramine (0.5 mg) was incubated with heart and with skeletal muscle aerobically, anaerobically (with a gas mixture of 95 per cent nitrogen and 5 per cent CO_2), and after the tissue was boiled for 5 minutes in saline. For heart muscle, 0.23 mg disappeared aerobically, 0.06 mg anaerobically, and tyramine was recovered quantitatively from the boiled tissue, which shows that the catalysts for these reactions are thermolabile and require oxygen. For skeletal muscle the respective values were 0.18, 0.07, and 0.03 mg. Since adsorption should occur anaerobically as well as aerobically, it cannot account for the disappearance of tyramine. The quantitative recovery of tyrosine and phenol from certain tissues that do not metabolize them indicates that these compounds also are not adsorbed to any extent. The gradual disappearance of tyramine from heart muscle with time also indicates that this compound is metabolized. Out of 1.0 mg added at the beginning 0.29 mg disappeared after 1 hour of incubation, 0.46 mg after 2.5 hours, and 0.6 mg after 4 hours.

Table I summarizes the results. Amino nitrogen values were only determined when 1.0 mg of tyrosine or tyramine was present in order that the difference between the control and experimental values should be as large as possible. In the case of the ileum the control values increased so much on incubation that the amino nitrogen contributed by the addition

TABLE I

Effect of Various Tissues on Tyramine, Tyrosine, and Phenol As Measured by Disappearance of Aromatic Hydroxy Group and in Case of First Two Compounds the Amino Group on the Side Chain

The tissues were incubated with the compounds for 4 hours at 37° in 95 per cent O₂, 5 per cent CO₂ (1.0 mg of tyrosine contains 0.077 mg of NH₂-N, 1.0 mg of tyramine, 0.103 mg of NH₂-N)

Tissue	Weight of slices	Compounds	Amount added	Amount recovered (as OH group)	Per cent disappeared	Amount recovered (as NH ₂ -N)	Per cent disappeared
	mg		mg	mg		mg	
Heart	184	Tyramine	0.5	0.23	54		
	160	"	1.0	0.36	64	0.046	45
	307	Tyrosine	0.5	0.49	2		
	246	"	1.0	0.92	8	0.085	0
Skeletal muscle	184	Phenol	0.5	0.47	6		
	228	Tyramine	0.5	0.24	52		
	215	"	1.0	0.50	50	0.029	72
	194	Tyrosine	0.5	0.33	34		
	267	"	1.0	0.71	29	0.079	0
Smooth muscle	228	Phenol	0.5	0.37	26		
	300	Tyramine	0.5	0.20	60		
	275	"	1.0	0.33	67		
	335	Tyrosine	0.5	0.30	40		
	287	"	1.0	0.71	29		
Kidney	337	Phenol	0.5	0.31	38		
	368	Tyramine	0.5	0.51	0		
	233	"	1.0	1.02	0	0.013	87
	364	Tyrosine	0.5	0.28	44		
	238	"	1.0	0.75	25	0.070	9
Liver	362	Phenol	0.5	0.42	16		
	235	Tyramine	0.5	0.48	4	0.000	100
	237	Tyrosine	0.5	0.22	56	0.062	19

of 1.0 mg of tyrosine or tyramine could not give a significant increase. The values are therefore not included. The figures listed in Table I were obtained on different days and different animals. Each is typical of a group of three to fifteen experiments. The weights of the slices were varied in some cases to illustrate certain points. The following facts about the metabolism of these compounds are evident from Table I. In the heart

the hydroxy and amino groups of tyramine disappear to about the same extent, indicating that both the amino group and the ring are split. The specificity of the reaction is, however, very marked, for in spite of almost twice the amount of tissue present the hydroxy and amino groups of tyrosine can be recovered almost quantitatively. Heart muscle is also unable to destroy phenol. Such specificity is not shown by either skeletal or smooth muscle. In the presence of the former the hydroxy groups of tyramine are attacked somewhat more readily than those of tyrosine and phenol. Tyramine is definitely deaminated, whereas the deamination of tyrosine does not occur. Smooth muscle attacks the hydroxy groups in a similar order, but the hydroxy groups of tyramine can be quantitatively recovered after incubation with kidney, although almost complete deamination occurs. Kidney reacts with *L*-tyrosine in exactly the opposite way, so that a significant number of hydroxy groups disappear but no deamination occurs. With liver the hydroxy group of tyrosine but not tyramine disappears but the deamination of the former is questionable. The inability of rat liver suspensions to deaminate tyrosine has been shown (6).

The effect of incubation of the muscle slices without addition of substrates on the amino nitrogen values and trichloroacetic acid-soluble chromogenic substances was determined. For 300 mg of skeletal muscle the initial amino nitrogen value was 0.05 mg and at the end of 4 hours incubation it had risen to 0.15 mg. The initial concentration of chromogenic substance measured against a tyrosine standard was 0.10 mg and at the end of 4 hours it was 0.19 mg. Since this increase in both values occurs anaerobically as well as aerobically, it can be attributed to the hydrolysis of protein or peptides, so that trichloroacetic acid-soluble substances are released. The increase in amino nitrogen in the ileum is more marked, rising from an initial value of 0.05 mg to 0.35 mg in 4 hours, and there is a corresponding increase in chromogenic substances. This is to be expected, as proteolytic enzymes must be present in considerable quantity. On the other hand neither the amino nitrogen nor the concentration of chromogenic substances rises in heart muscle on incubation. It should be pointed out that these increases in skeletal and smooth muscle do not interfere with the determination of the metabolism of the added compounds, because simultaneous controls were always used and the values expressed as differences.

DISCUSSION

Tyramine is readily metabolized by tissues *in vitro*. With the exception of kidney and liver, which only deaminate it, tissue slices appear to break the ring as well, for there is a significant disappearance of estimable hydroxy

groups which is not caused by conjugation. Whether tyramine is the normal substrate for the enzyme systems present in these tissues is impossible to say but it seems probable that related pressor amines can also be attacked in a similar way. Although the heart is unable to destroy tyrosine, the other tissues are probably able to break the ring but with the possible exception of liver no deamination occurs. As has been previously shown (5), liver is able to oxidize and conjugate phenol. Heart is unable to do either but skeletal and smooth muscle can apparently oxidize but not conjugate it. This inability of muscle to conjugate phenol was also shown by Arnolt and de Meio (7) who used diaphragm. They found that intestinal mucosa can conjugate phenol and this may be the locus of the formation of epinephrine sulfate when the hormone is taken by mouth (8). The results of Barac (9) who found that phenol is conjugated by the eviscerated dog are not explained by these *in vitro* experiments.

SUMMARY

1 Heart, skeletal, and possibly smooth muscle of the rat is able to deaminate tyramine and because of the disappearance of estimable hydroxy groups can presumably also break the ring. Kidney and liver are only able to deaminate it.

2 Because of the disappearance of estimable hydroxy groups all these tissues with the exception of heart can presumably break the ring of tyrosine. With the possible exception of liver no deamination occurs.

3 Heart is unable to destroy phenol. Skeletal and smooth muscle can oxidize but not conjugate it, and liver as previously shown is able to oxidize and conjugate it. When phenol is added to kidney, only a small percentage disappears.

4 None of these reactions occurs anaerobically or in the presence of boiled tissue.

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A PHOTOMETRIC ADAPTATION OF THE SOMOGYI METHOD FOR THE DETERMINATION OF GLUCOSE

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The reliability of the various Somogyi-Shaffer-Hartmann (1, 2) copper reagents for glucose determination in biological material has been established. Adaptation of these reagents to colorimetric use may be accomplished by omission of the iodide and iodate in their preparation, since these interfere with the molybdate color reagents. This omission produces no especial change in the character of the reagents. KI, however, inhibits the autoreduction of the copper and in its absence an unstable reagent results. Nevertheless, if the copper is added to the rest of the reagent on the day of its use, this difficulty is avoided.

When the Somogyi micro reagent (2) is used in this way with almost any of the various phosphomolybdate reagents, very satisfactory proportionality is found between color density and glucose taken over a wide range of values. However, all of the phosphomolybdate reagents tried left much to be desired in reproducibility from time to time and lacked the desired stability of color.

We therefore tried various color reagents, which led to the development of a new arsenomolybdate reagent. When this reagent was used with Somogyi's micro reagent, it gave satisfactory stability and reproducibility of color. By this means it has been possible to utilize the copper reagents in a photometric procedure for practically all the uses to which the titrimetric procedures are adapted. These include tissue sugar, glycogen, urine reduction equivalent, maltose, glucuronic acid, etc. However, diastase determinations have not been successful because of the effect of the undigested starch on the clarity of the final colored solution.

The reactions involved in the molybdenum blue reaction are uncertain and beyond the scope of this report. Woods and Mellon (3) discuss and give references to the various interpretations of the reaction.

Reagents—Analytical reagent grade or the equivalent.

1 Copper Reagent A. Dissolve 25 gm of Na_2CO_3 (anhydrous), 25 gm of Rochelle salt, 20 gm of NaHCO_3 , and 200 gm of Na_2SO_4 (anhydrous) in about 800 ml of water and dilute to 1 liter. Filter if necessary.¹ This

¹ The effective linearity of results in the very low range of reduction has been improved over that secured in the ordinary Somogyi micro blood sugar procedure. This

solution should be stored where the temperature will not fall below 20° a sediment may form after a few days This may be filtered off without detriment to the reagent

Copper Reagent B 15 per cent $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ containing one or two drops of concentrated sulfuric acid per 100 ml

2 Arsenomolybdate color reagent Dissolve 25 gm of ammonium molybdate in 450 ml of distilled water, add 21 ml of concentrated H_2SO_4 , mix, add 3 gm of $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in 25 ml of H_2O , mix, and place in an incubator at 37° for 24 to 48 hours Fig 1 shows the progress of formation of the chromogenic compound during incubation at 37° If a reagent is needed quickly, an alternative procedure is to heat to 55° for about 25 minutes However, stirring must be adequate to prevent local overheating, otherwise decomposition of the chromogen may occur This is accompanied by the precipitation of a bright yellow compound The first procedure has been uniformly successful and is the recommended one, the second is inconvenient, and with certain preparations of sodium arsenate yields reagents which, though useful, are inferior in potential color development to those prepared by the first procedure This reagent should be stored in a glass-stoppered brown bottle ²

3 5 per cent $\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$

4 Approximately 0.3 N $\text{Ba}(\text{OH})_2$ The zinc and barium solutions should be adjusted so that 5 ml of zinc require between 4.7 and 4.8 ml of barium to produce a definite pink to phenolphthalein The zinc should be diluted to 20 or 25 ml with H_2O and the $\text{Ba}(\text{OH})_2$ added dropwise with constant mixing during the titration Store in a bottle protected by soda lime from the carbon dioxide of the air It is convenient to have an arrangement for direct delivery of the $\text{Ba}(\text{OH})_2$ into a 5 ml burette graduated to 0.01 or 0.02 ml

is done by using 1 volume of 1.20 blood filtrate per volume of Reagent A instead of 1 volume of 1.40 In order to extend the useful range of the reagent to compensate partially for this change, 0.6 per cent copper sulfate is used instead of 0.4 per cent This procedure has an additional advantage in that a smaller amount of reagent will suffice, and, consequently, the blank (which arises chiefly from the reagents other than copper sulfate) will be reduced correspondingly

² Reagents prepared by either of the procedures described probably will have considerably more of the active chromogen per ml than will be needed for 0.3 mg of glucose (our arbitrary upper limit of estimation) To economize on the reagent, one may determine in a titration against the maximum amount of glucose which it is desired to estimate how much reagent is required to give maximum color On the basis of this, the reagent may be diluted with 1.5 N H_2SO_4 to allow about a 20 per cent excess concentration of the chromogenic compound over the maximum needed

Procedure

The blood filtrates are prepared as follows.³ Add 1 volume of blood to 15 volumes of water, mix, add 2 volumes of $\text{Ba}(\text{OH})_2$, mix, and after the mixture has turned brown add 2 volumes of ZnSO_4 and mix. After a few minutes the mixture may be filtered, somewhat more filtrate may be secured by a preliminary centrifugation. For finger-tip blood, one may wash 0.1 ml of blood from a pipette calibrated "to contain" into 1.5 ml of water contained in a small vial or test-tube. After 0.2 ml each of $\text{Ba}(\text{OH})_2$ and ZnSO_4 is added as described above, the mixture is centrifuged. The filtrate is then drawn into a 1 ml pipette tipped with washed cotton.

1 ml of filtrate is pipetted into a narrow test-tube graduated at 25 ml. The ordinary Folin-Wu blood sugar tubes are convenient but not essential.⁴ 1 ml of a mixture (prepared the day of use) of 25 parts of Reagent A to 1 part of Reagent B is added. Since this latter volume is not critical, a burette or measuring pipette may be used. 1 ml portions of appropriate standards and 1 ml of distilled water, to serve as a blank, are set up in the same way. The solutions are mixed and heated for 20 minutes in a boiling water bath. At the end of 20 minutes the tubes are cooled in a pan of cold water. 1 ml of the arsenomolybdate reagent is then added to each, a measuring pipette is convenient and adequate for this measurement. The color develops very rapidly and will be completed by the time thorough mixing and evolution of CO_2 are completed. The mixture is then diluted to the mark, mixed, and read in a photoelectric colorimeter at 500 or 520 $m\mu$. The photometer is adjusted so as to read 100 per cent transmission through the blank. The color is very stable and may therefore be read at convenience. The stability of the color is absolute and not relative, the density of the blanks as well as of the more deeply colored solutions remains unchanged with time.

It has been our practice to run duplicate determinations on all blood

³ The deproteinization procedure given is one suggested to us by Dr. Somogyi several years ago. It has been well known (Benedict (4), Somogyi (2)) that the ZnSO_4 - NaOH deproteinization procedure leaves a small amount of Zn remaining in the filtrate and that this trace apparently accelerates the reoxidation of cuprous oxide. The extent of this is small and relatively unimportant in a macro blood sugar estimation, but decidedly significant in a microdetermination. We had encountered this difficulty and devised a procedure similar to but more cumbersome than the one Somogyi was using to overcome the same difficulty. We have found his method a very useful technique for various purposes, since it has the advantage of yielding a filtrate practically free of the deproteinizing reagents.

⁴ The high Na_2SO_4 concentration of the reagent gives adequate protection against reoxidation for most purposes, so that neither the constricted Folin-Wu tube nor covered tubes are essential. However, if high accuracy with quantities of glucose below 5 γ is needed, these precautions should be taken.

sugars and to run each urine determination at two levels of dilution. In our records for a 6 months period, covering over 2000 determinations, a deviation of 3 per cent between duplicates is rare. In most cases the spread was less than 1.5 per cent of the amount determined. We routinely carry through one pair of standards and blanks with each set and once each day three pairs of standards are included, for this we use 0.05, 0.15, and 0.3 mg of glucose per ml, corresponding to 1 ml of a 1:20 dilution of blood containing 100, 300, and 600 mg per 100 ml respectively. The variation

TABLE I

Optical Density (Log I_0/I) at 500 $M\mu$ at Various Intervals after Color Development

Glucose taken mg	Time			
	0	25 min	100 min	18 hrs
0.050	0.169	0.169	0.169	
0.300	0.979	0.979	0.987	
0.025	0.089			0.087
0.050	0.175			0.173
0.150	0.520			0.522

TABLE II

Relationship between Density (Log I_0/I) and Glucose Taken (Average of Duplicate Determinations)

Glucose taken, ϵ mg	Optical density, d	$\frac{\epsilon}{d}$
0.005	0.0175	0.286
0.025	0.0865	0.289
0.050	0.1760	0.284
0.150	0.5140	0.292
0.300	1.015	0.295

in the density values for these standards from one batch of reagents to another is not essentially greater than that encountered from day to day with the same reagents. The maximum spread of the density values has been about 6 per cent. Usually the density coefficient of the standards can be predicted with a 1 or 2 per cent precision.

Representative data indicating the stability of the color developed are given in Table I. The optical density was occasionally checked as late as 3 days after development, when only small changes from the initial values were found.

Table II shows a typical calibration indicating the essential proportionality between optical density and glucose taken

The color densities were read in a photoelectric spectrometer (based on a Gaertner model 227 monochrometer) in Evelyn colorimeter tubes at a wave-length of $500\text{ m}\mu$, with slit widths of 0.1 mm . However, other laboratories have used Filter 520 with the Evelyn colorimeter successfully for this purpose. At these wave-lengths the light absorption is far from the maximum, which lies at $660\text{ m}\mu$ (Fig 1). The wave-length $500\text{ m}\mu$ was chosen because it represented a satisfactory compromise between the sensitivity desired

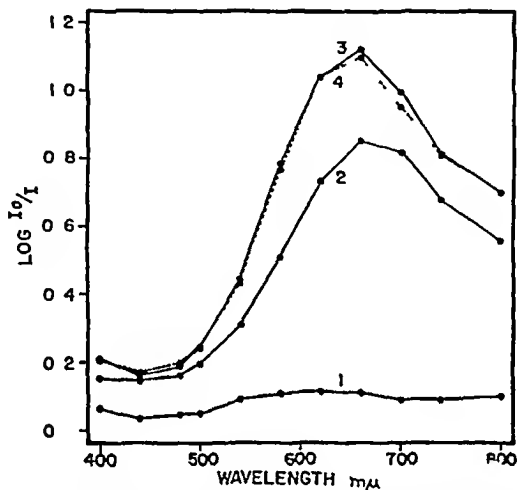


FIG 1 Development of the arsenomolybdate chromogenic compound at 37° . 1 ml of a 1:10 dilution in $0.5\text{ N H}_2\text{SO}_4$ of the reagent described in the text was added to excess cuprous oxide. The monochromator slits were set at 0.05 mm for wave lengths of $600\text{ m}\mu$ and above, and at 0.10 mm for wave lengths below $600\text{ m}\mu$. Curve 1, 0 time, Curve 2, 12 hours, Curve 3, 24 hours, Curve 4, 48 hours

and the advantages gained by reducing to a minimum the effect of variation in such factors as blank due to reagents, reoxidation of cuprous oxide, etc. The sensitivity can be increased over 4 times merely by reading the light transmission at $660\text{ m}\mu$.

Use of the color reagent for measurement of the reduced copper formed in the Somogyi reagent is regarded merely as an alternative to iodometric titration. Within the limits of error of the two procedures, equivalent results have been secured in all applications but measurement of diastatic

activity The characteristics of the reagents are available in the original articles (1, 2)

Thirty-nine blood specimens run in parallel by the above procedure and by the Somogyi titrimetric method showed an average of 155.7 mg per cent for the titration against 155.9 mg per cent for the photometric technique. The standard error of the difference of the means was 0.007. The blood sugar ranged from 45 to 585 mg per 100 ml and included specimens from normal and diabetic owls, dogs, monkeys, and humans. The same blood filtrates were used for both determinations.

Although we have not used the procedure with a Duboscq colorimeter, there appears to be no reason why it should not offer some advantages over the usual methods. A yellow filter or light source such as that used by Folin (5) would probably facilitate the comparison of standard with unknown.

SUMMARY

A photometric method has been described for the estimation of glucose (or reduction equivalent) with copper reagents and an arsenomolybdate reagent. The optical density of the color developed is proportional to the glucose taken and is stable over long periods of time.

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THE COMPOSITION OF THE TISSUE PROTEINS OF THE RABBIT AS INFLUENCED BY INANITION AND THE HEPATOTOXIC AGENTS, HYDRAZINE AND PHOSPHORUS

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Recent investigations of tissue metabolism in which isotopic nitrogen (N^{15}) has been used as a marker have indicated that a "rapid and continuous chemical regeneration of the cell proteins is a general characteristic of living matter" (1). Despite this striking and continuous "chemical activity" of the organ proteins, it is believed that these processes "lead to no final quantitative or qualitative changes" (2) in the composition of the proteins of the tissues. This is in confirmation of the older belief in the constancy of composition of the structural elements of protoplasm, well illustrated by the statement of Osborne and Mendel (3), "That the tissues either form a typical protoplasmic product, or none at all, now seems to be axiomatic in physiology."

This point of view is in sharp contrast to the hypothesis that tissue proteins are variable in composition. It is stated that, in the white rat, quantitative changes in the amino acids of the proteins of the tissues may be effected readily by "protein starvation" (4), by changes in diet, by infections, by toxic agents, by the administration of hormones, and by irradiation (5). In particular, changes in the content of tryptophane, tyrosine, and lysine (4), of cystine and tryptophane (5), and of arginine (6) have been observed. Since the present investigation is concerned only with tissue proteins, the rather extensive literature relating to possible alterations in the composition of the serum proteins will not be reviewed here. This discussion will serve to illustrate the conflict between the observations of Roche (4) and Cahn and Bonot (6), of Schenck and Wollschitt (5), and the older hypothesis of the constancy of the composition of proteins which are essential components of tissues. Lee and Lewis (7) analyzed the mixed proteins of liver, kidney, and muscles of young white rats fasted and fed adequate mixed diets and also diets varying in their content of cystine, one of the amino acids for which lability in tissue proteins has been claimed. No evidence to support the hypothesis that the composition of the proteins of these tissues was influenced by dietary factors, as maintained by Schenck and Wollschitt (5), was obtained. The present paper is concerned with similar studies on young rabbits in a condition of normal nutrition and in

inanutlon Since various toxic agents have been stated to alter the structural pattern of tissue proteins (5) and since the liver is an organ importantly concerned in protein metabolism, it was considered of particular interest to study the effect of intoxication produced by two hepatotoxic agents, hydrazine and yellow phosphorus, on the composition of tissue proteins Under the conditions of the present experiments, no alterations in the quantitative composition of the total tissue proteins of liver, muscle, or kidney, as far as concerns the yield after complete hydrolysis of the amino acids, tyrosine, tryptophane, or cystine, were observed

EXPERIMENTAL

Young albino rabbits in litter units were selected as experimental animals Each litter of six to seven animals, usually 6 to 8 weeks of age when received in the laboratory, was divided into a control and an experimental group, each consisting so far as possible of equal numbers of animals of the same sex The animals were fed a commercial mixed food for at least a week prior to the experimental periods The usual procedure was to kill one animal of each group (control and experimental) in the same week, the age differences between any pair of animals being at most 3 to 4 days As a rule, two animals, one from each group, were killed each week The first animals of each litter, when sacrificed, were thus about 9 to 10 weeks of age, and the last from 12 to 13 weeks In a few instances, the animals were maintained on the commercial mixed food for 6 to 8 weeks before the experiments were begun The adequacy of this diet was shown by the good growth of the young animals under our laboratory conditions Although the litters used were separated into groups according to sex, no difference in the results attributable to this factor was observed

In the first series, a comparison between the proteins of the tissues of well fed and fasted animals was made The control groups were fasted 5 days, while the experimental groups received the usual diet *ad libitum* for the same period In the second series, both the control and experimental groups fasted for 5 days, but the animals of the experimental group received 25 mg of hydrazine (as the hydrochloride) per kilo of body weight subcutaneously on the 4th and 5th days of fasting The animals were killed at the end of the 5th day¹ In the third series, yellow phosphorus, suspended and emulsified in warm corn oil, was similarly injected subcutaneously into the animals of the experimental group Amounts of this solution equivalent to 3.5 mg of phosphorus per kilo of body weight were

¹ In the blood of the rabbits treated with hydrazine, varying degrees of hemipia were noted Although histological examinations of the livers were not made, the gross picture was that of moderate fatty infiltration

injected on the 3rd and 4th days of fasting, and half this amount on the 5th day² The control groups fasted 5 days, as in the other series

At the conclusion of the final day of the 5 day period, as much blood as possible was removed from the heart with a needle and syringe The animal was then stunned by a blow on the neck and after exposure of the jugular vein killed by exsanguination Liver, kidneys, and skeletal muscle of the fore and hind limbs were removed immediately and the protein was prepared from these tissues by the method of Janney (8)

The livers in these experiments always contained small but variable amounts of residual blood Although the amount of this was believed to be too small to alter significantly the results of the analyses, it was considered desirable to remove as much blood as possible by perfusion of the livers, and to compare the liver proteins thus obtained with those from liver prepared in the usual way The procedure used for perfusion was essentially that of Luck (9) except that no phosphate was added to the saline used

The time elapsing between the beginning of the perfusion and the sampling for the preparation of the protein was approximately 1 hour The muscles and kidneys obtained in these experiments were also analyzed, but perfusion of these organs was not attempted

The protein obtained from the perfused livers was less highly pigmented than the protein obtained from livers which were not perfused Analyses showed essentially the same composition of the two preparations of liver protein

The methods of analysis of the tissue proteins were the same as those previously described (7) Duplicate hydrolyses of the proteins were possible except in a few cases in which the amount of protein available was insufficient (kidneys) With each hydrolysate duplicate or triplicate determinations were carried out All results are calculated on an ash-free, moisture-free basis

In Table I are presented the data obtained in the series in which the total coagulable proteins of the tissues of well fed and fasted rabbits were analyzed In addition, the similar analyses for rat muscle (7) and the composition of myogen and myosin (10) of rabbit muscle, the two best characterized proteins of this tissue, are given It will be seen that there are no significant differences between the two sets of values, a finding which confirms previous observations on rats from this laboratory (7) The low total nitrogen content of the "protein" prepared from the liver of the well

² Pathological examination of the livers of some of these animals showed degenerative changes characteristic of phosphorus poisoning We wish to express our appreciation to Professor Carl V Weller, of the Department of Pathology, for his helpful cooperation in examining the tissues

fed animals by the application of the method of Janney is probably due to the association of the liver proteins with some other protoplasmic component or components in well fed animals. Similar low nitrogen values were reported by one of us (7) in the analyses of liver protein from well fed rats. The existence of a complex of protein and liver glycogen has been postulated (11, 12). The nitrogen contents of the proteins of other tissues and of the livers of fasted animals are satisfactory. The ratio of the nitrogen content to that of the sulfur of the liver protein of the well fed rabbits is within the anticipated range of values and indicates that, whatever the associated substance may be, it probably contains no sulfur or nitrogen.

TABLE I

Composition of Tissue Proteins of Well Fed and Fasted Rabbits

The figures represent the averages of analyses of proteins prepared from the tissues of eight animals of each group and are calculated on an ash free, moisture free basis

Tissue	Nutrition	Total N	Total S	N/S	Cystine N of total N	Cystine S of total S	Tyrosine N of total N	Tryptophane N of total N
		per cent	per cent		per cent	per cent	per cent	per cent
Liver	Fed	12.31	0.82	15.0	1.56	53.8	2.22	1.15
"	Fasted	15.85	1.03	15.4	1.41	50.0	2.09	0.96
Kidney	Fed	16.12	1.08	14.9	1.30	41.5	1.89	0.90
"	Fasted	16.13	1.06	15.2	1.42	48.0	2.03	1.01
Muscle	Fed	15.95	0.99	16.1	1.01	37.3	1.89	0.89
"	Fasted	16.14	1.02	15.8	1.06	38.6	1.93	0.83
Muscle* (rat)	"	16.2	0.99	16.3	0.80	30.3	2.13	1.35
Myogen†		16.6	1.29	12.9	1.37	40.5	1.95	1.20
Myosin†		16.7	1.10	15.2	0.53	18.6	1.56	0.67

* Total muscle protein from the data of Lee and Lewis (7).

† Purified protein fractions of rabbit muscle, calculated from the data of Bailey (10).

The analyses of the proteins of the tissues of animals which were fasted and then received hepatotoxic agents, when compared with the analyses of the fasted control groups in each case, also showed no differences in the yield of the amino acid studied (Table II). No evidence is to be obtained from any of the data here presented that the total coagulable protein of the tissues is as easily altered in its content of cystine, tyrosine, or tryptophane, as is suggested by Schenck and Wollschitt (5). The data do not rule out possible changes in the distribution of various fractions of liver protein (9), but the composition of the total coagulable protein as prepared by the method of Janney remains unchanged. The data are in confirmation of our previous studies with white rats. It is of interest to note that Wake

man (13), in 1905, analyzed the livers of dogs receiving hepatotoxic agents and believed that the data suggested a tendency "toward a diminution of the hexone bases as a whole" and, in particular, the arginine content. Subsequently in studies of the liver of dogs similarly treated and in hepatic disease of man (14), he observed changes in the content of hexone bases which were "as a rule comparatively slight" and probably within the limit

TABLE II

Composition of Tissue Proteins of Fasted Rabbits and of Fasted Rabbits Treated with Hepatotoxic Agents (Hydrazine, Yellow Phosphorus)

All values are averages for the group and are calculated on an ash free, moisture free basis

Tissue	Group No	Nutrition	No of animals	Total N	Total S	N S	Cystine N of total N	Cystine S of total S	Tyrosine N of total N	Tryptophane N of total N
				per cent	per cent		per cent	per cent	per cent	per cent
Liver	1	Fasted	5	15.61	1.07	14.6	1.57	51.9	2.12	0.97
		Hydrazine	7	15.81	1.05	15.0	1.48	50.9	2.11	1.02
	2	Fasted	4	15.55	1.09	14.3	1.43	46.3	2.08	1.04
		Phosphorus	6	15.86	1.14	13.9	1.34	42.4	2.04	1.03
	3*	Fasted	3	15.99	1.07	14.9	1.34	43.7	2.04	1.08
		Phosphorus	3	15.95	1.15	13.8	1.41	44.2	1.99	1.15
Muscle	1	Fasted	5	16.10	1.03	14.9	1.12	37.9	2.00	0.94
		Hydrazine	7	16.07	1.06	15.2	1.12	38.1	2.06	0.87
	2	Fasted	4	16.10	1.06	15.2	1.06	41.3	2.00	1.00
		Phosphorus	6	15.84	1.05	15.1	1.14	38.0	2.03	1.01
	3*	Fasted	3	15.96	1.06	15.0	1.01	35.2	2.03	1.14
		Phosphorus	3	15.82	1.12	14.1	1.27	39.7	1.98	1.08
Kidney	1	Fasted	5	16.05	1.14	14.1	1.53	48.2	2.17	0.96
		Hydrazine	7	15.98	1.12	14.3	1.45	46.8	2.08	1.01
	2	Fasted	4	16.06	1.09	14.7	1.40	38.6	2.11	1.08
		Phosphorus	6	15.80	1.11	14.2	1.49	47.7	2.14	1.10
	3*	Fasted	3	15.69	1.15	13.6	1.50	45.1	2.03	1.24
		Phosphorus	3	15.68	1.22	12.8	1.50	43.7	2.03	1.24

* The livers of these animals were perfused as described in the text

of individual variation. These data were obtained on hydrolysates of liver and not on the hydrolysates of the isolated proteins.

It is realized that the methods for the analysis of various amino acids in protein hydrolysates are imperfect, but it is believed that they are sufficiently accurate to permit comparisons when materials of similar origin and preparation are analyzed.

SUMMARY

1. Proteins prepared from the liver, muscle, and kidney of young rabbits by the method of Janney (8) (*i.e.*, total heat-coagulable protein) have been

analyzed for total nitrogen and sulfur, and for the amino acids, cystine, tyrosine, and tryptophane

2 The composition of these tissue proteins was found to be essentially the same (a) in well fed and fasted (5 days) animals and (b) in fasted animals in comparison with animals receiving the hepatotoxic agents, hydrazine and phosphorus

3 The data fail to offer support to the hypothesis of Schenck and Wollschütt (5) that the composition of the tissue proteins is readily altered by changes in the metabolic state of the animal

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L-AMINO ACID OXIDASE OF *PROTEUS VULGARIS*

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Proteus vulgaris contains an enzyme which catalyzes the oxidation of some eleven amino acids to their corresponding keto acids with liberation of ammonia. The present communication deals with the properties of this enzyme both in the intact bacterial cell and in cell-free extracts.

Bernheim *et al.* (1) were the first to observe the amazing ability of *Proteus vulgaris* to oxidize practically all the known natural amino acids. We have confirmed their observations when we used bacterial suspensions which were freshly harvested. However, as the age of the bacterial suspension increased, the number of amino acids attacked dwindled. Thus a suspension which had been kept at 0° for 2 weeks oxidized only eleven of the twenty-two amino acids which were attacked originally. Further aging of the suspension did not reduce the list, although it resulted in some rearrangement in the order of velocities of oxidation. This was particularly true in the case of isoleucine. Eventually no further change was observed either in the number of amino acids which were oxidized or in the relative velocities.

The above observations are readily explained in the following way. There are at least several enzymes which are involved in the oxidation of twenty-two amino acids by young suspensions of *Proteus vulgaris*. All but one of these enzymes are relatively unstable and disappear in turn as the bacterial suspension ages. Eventually only one enzyme survives, *viz.*, the one which is the subject of the present communication and which we shall call the *l*-amino acid oxidase of *Proteus vulgaris*. This unspecific name for the enzyme is inadequate either as far as distinguishing it from other enzymes which attack *l*-amino acids or as far as grouping it with enzymes from other sources with similar, if not identical, properties. However, a rational nomenclature will have to await more information on the specificities of the various enzymes which attack *l*-amino acids. In the present communication the term *l*-amino acid oxidase will be reserved for the particular enzyme of *Proteus vulgaris* whose properties are described below. Any of the other enzymes which oxidize *l*-amino acids will be characterized either on the basis of their origin, *e.g.* the *l*-oxidase of rat kidney, or on the basis of some particular substrate which they oxidize readily, *e.g.* the alanine oxidase.

We do not have adequate data to decide precisely how many amino acid oxidases are concerned altogether and whether the mechanism of oxidation

is identical in all cases. The glycine, serine, and proline oxidases are least stable and disappear first. The alanine, valine, aspartic, and glutamic oxidases are somewhat more stable and survive storage at 0° for 1 week. Increasing the temperature of storage of the bacterial suspension hastens the decay of the fragile amino acid oxidases¹. Thus, storage for 24 hours at 38° is roughly equivalent to storage for about 1 week at 0°.

The *l*-amino acid oxidase can be obtained cell-free by disintegrating the bacterial cell with supersonic vibrations. When young bacterial suspensions are thus treated, the extracts contain enzymes other than the *l* amino acid oxidase which can oxidize certain natural amino acids. However, by the method of purification (to be described later) the *l*-amino acid oxidase is completely separated from these other enzymes. We have thus been able to study the *l*-amino acid oxidase uncomplicated by the presence of other amino acid oxidases, both in the intact cell and in cell-free extract.

Specificity of Substrate—The enzyme oxidized all the unsubstituted mono carboxylic monoamino, primary amino acids of the *l* series with the exception of alanine and valine (cf Table I). The β -hydroxy, dicarboxylic, and diamino amino acids (arginine excepted), proline, hydroxyproline, and cysteine (or cystine), glycine, phenylglycine, and its *N* acetyl derivative were not oxidized. β -Amino acids such as β -alanine and isoserine, amines (histamine and tyramine), peptides (reduced glutathione, glycylglycine, and leucylglycine), asparagine, glutamine, and *N* methylamino acids such as *N*-monomethylleucine, *N*-monomethylmethionine, and *N*-monomethyl histidine were all not oxidized by the oxidase.

Marked stereochemical specificity was shown by the enzyme. Only the *l* forms of the amino acids were attacked. The *d* forms which accumulated during the oxidation of *dl*-amino acids did not inhibit the oxidation of the corresponding *l* forms. We have taken advantage of this configurational specificity to prepare *d*-amino acids from the *dl* forms. In the experimental section details are given only for the preparation of *d*-methionine, although *d*-leucine and *d*-phenylalanine were also prepared by the same method.

The relationship between rate of oxidation and number of carbon atoms in the aliphatic side chain of an amino acid of a given series is shown in Table II. The relationship was tested in three series of amino acids which we shall refer to respectively as unbranched, branched, and phenyl-substituted. The unbranched series include only straight chain, unsubstituted amino acids like glycine, alanine, etc. The so called branched series is limited to those amino acids which correspond to the aliphatic fatty acids of the iso series, such as isovaleric acid and isocaproic acid. It would not, for ex

¹ The term amino acid oxidase is used loosely here to include enzymes of all types which facilitate the oxidation of amino acids without necessarily implying oxidative deamination.

ample, include isoleucine, which is a β -methyl derivative of normal valeric acid. Finally, the phenyl-substituted series includes amino acids which have a phenyl group in ω position, like phenylglycine, phenylalanine, etc.

TABLE I

Relative Velocities of Oxidation of Amino Acids by Cell Suspensions and Cell Free Extracts

The velocity of oxidation was measured manometrically by the rate of oxygen uptake in the presence of excess of substrate. Each manometric cup contained 0.5 cc of bacterial suspension (4 mg, dry weight) or 0.1 cc of the cell free preparation (1.1 mg, dry weight), amino acid equivalent to 0.5 cc of 0.1 M amino acid, and 0.05 M phosphate buffer of pH 7.1 to make a final volume of 3 cc. Alkali in center well, 38°.

Amino acid	Relative velocities*		
	Fresh suspension	Aged suspension	Cell free extract
<i>dl</i> -Phenylalanine	100	100	100
<i>l</i> -Tyrosine	99	54	62
<i>l</i> -Leucine	93	97	91
<i>dl</i> -Isoleucine	89	31	15
<i>l</i> -Methionine	87	91	65
<i>l</i> -Tryptophane	75	94	88
<i>l</i> -Histidine	73	25	33
<i>dl</i> -Norleucine	80	90	108
<i>dl</i> -Norvaline	90	87	60
<i>dl</i> -Aminobutyric acid	72	19	12
<i>l</i> -Arginine	42	23	30
<i>dl</i> -Serine	63	0	0
<i>l</i> -Aspartic acid	57	0	0
<i>l</i> -Glutamic acid	54	0	0
<i>dl</i> -Alanine	45	0	0
<i>dl</i> -Valine	24	0	0
<i>l</i> -Proline	22	0	0
<i>dl</i> -Threonine	22	0	0
<i>l</i> -Ornithine	14	0	0
<i>l</i> -Lysine	14	0	0
Glycine	10	0	0
<i>dl</i> -Phenylglycine	0	0	0

* Relative velocities were calculated on the basis that the rate of oxidation of *dl*-phenylalanine was 100 in each case. Velocities are expressed, therefore, as percentages of the standard velocity.

There is apparently in each series a definite chain length at which the greatest velocity of oxidation is attained. Below or above this critical length the velocities fall off sharply. In the unbranched series the amino acid with 6 carbon atoms was oxidized most rapidly. As the number of carbon atoms

decreased below 6, the velocity fell off and reached zero for the 3 carbon atom compound (alanine). The velocity for the 8-carbon atom compound (aminocaprylic acid) was half that for the 6-carbon atom compound (nor-leucine). The picture was essentially the same in the phenyl-substituted series. Phenylalanine, with 3 carbon atoms in the aliphatic side chain, was most rapidly oxidized. Phenylglycine, the lower homologue, was not oxidized at all, whereas phenylaminobutyric acid was oxidized at a rate less than one-fourth that of phenylalanine. Only two compounds were avail-

TABLE II

Relationship between Length of Straight Chain of Amino Acid and Rate of Oxidation

Each manometer cup contained in addition to the amino acid 0.2 cc. of bacterial suspension which had been stored 2 months at 0° and 0.1 M phosphate buffer of pH 7.1 to make a final volume of 3.4 cc. Alkali in center well, 38°

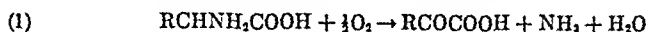
Series	Substrate	No. of carbon atoms in straight chain of amino acid	O ₂ per 5 min	
			M/180*	M/36
Unbranched	<i>dl</i> Alanine	3	0	0
	<i>dl</i> -Aminobutyric acid	4	4	8
	<i>dl</i> Aminovaleric "	5	17	25
	<i>dl</i> Aminocaproic "	6	20	36
	<i>dl</i> Aminocaprylic "	8	10	
Phenyl substituted			M/340	M/260
	<i>dl</i> Phenylglycine	2	0	0
	<i>dl</i> Phenylalanine	7	16	20
	<i>dl</i> Phenylaminobutyric acid	4	4	3
Branched			M/60	M/20
	<i>dl</i> Valine	4	0	0
	<i>l</i> Leucine	5	23	50

* The molarity refers to the initial concentration of the substrate in the manometer cup

able for testing the relationship in the branched series, viz., valine and leucine. Leucine was oxidized, whereas valine was unattacked.

Comparison of the rates of oxidation of the eleven substrates of the enzyme when the enzyme is present in the "aged" cell and when isolated and purified disclosed no appreciable change in the relative velocities provided the precaution of complete "aging" was taken to eliminate the action of enzymes other than the *l*-amino acid oxidase under study. This evidence is consistent with the assumption that one enzyme catalyzes the oxidative deamination of all eleven substrates, but it still does not exclude the possibility that several enzymes with rather similar properties are involved.

Products of Reaction—The oxidation of all substrates of the *l*-amino acid oxidase follows the equation



The keto acids corresponding to phenylalanine, methionine, isoleucine, leucine, tyrosine, tryptophane, norleucine, norvaline, histidine, and arginine have been isolated in good yields in the form of their 2,4-dinitrophenylhydrazones. Considerable interest attaches to the keto acid of arginine, which previously had never been prepared synthetically or isolated from natural sources.

According to Equation 1, for each atom of oxygen absorbed, 1 molecule of amino acid is oxidized, with the formation of 1 molecule each of keto acid and ammonia. The relationship between oxygen uptake and formation of both keto acid and ammonia is as follows

Substrate	O ₂ uptake	NH ₃	Keto acid	O NH ₃ keto acid		
	microatoms	micromoles	micromoles			
<i>dl</i> Phenylalanine	34.8	34.8	35.5	1	1.00	1.02
<i>l</i> Leucine	38.3	42.3	37.4	1	1.10	0.98

Within the limits of experimental error, the data for the oxidation of leucine and phenylalanine satisfy the requirements of Equation 1.²

The *l*-amino acid oxidase of rat kidney produces H₂O₂ during its reaction with molecular oxygen (2), whereas the *l*-amino acid oxidase of *Proteus vulgaris* does not. This difference is the most significant one yet found. Since the demonstration of H₂O₂ formation by the animal enzyme was possible only in purified preparations which were free of catalase, we were at first inclined to the view that H₂O₂ formation by the *Proteus* enzyme was obscured by the action of catalase. However, even when the level of active catalase was reduced to negligible proportions, either by purification or by the addition of selective inhibitors, the evidence was still unequivocal that

² The quantitative experiments were carried out with the purified enzyme rather than cell suspensions, since the latter slowly oxidized some of the keto acids. In all but one case the rate of oxidation of the keto acids by cell suspensions was small compared to the rate of its formation from the corresponding amino acid. Nevertheless, oxidation of keto acid was not insignificant and had the effect of obscuring the quantitative relationships between oxygen uptake and formation of keto acid. When fresh cell suspensions were allowed to act on small amounts of alanine, serine, threonine, glutamic acid, and aspartic acid (none of which is a substrate of the enzyme), the oxygen uptake far exceeded the theoretical quantity required for keto acid formation. This indicated that the keto acids were further oxidized. Indeed, pyruvic acid and α -ketoglutaric acid when added to such suspensions were rapidly oxidized. α -Aminobutyric acid was the one amino acid attacked by the *l*-amino acid oxidase whose corresponding keto acid was rapidly oxidized by cell suspensions. However, α -ketobutyric acid was stable in the presence of the isolated enzyme.

1 atom of oxygen was taken up per molecule of amino acid and not 2 atoms of oxygen, as would be required if H_2O_2 were formed. Furthermore, the delicate test for formation of H_2O_2 worked out by Keilin and Hartree (3) was consistently negative when carried out on the complete enzyme system.

Preparation and Properties of Enzyme—Cell suspensions of *Proteus vulgaris* were disintegrated rapidly by exposure to ultrasonic vibrations (600 kc). The essential details of the oscillator and the conditions for disintegration are given in the experimental section. After treatment, the suspension was centrifuged with hard packing of the sediment to remove all intact cells as well as cellular debris and ghost cells. The supernatant fluid was opalescent and pale yellow in color. The sediment consisted of two layers (1)

TABLE III
Fractional Centrifugation of Enzyme

Stage	Gravitational field	Centrifugation time	QO ₂ in presence of phenylalanine
	<i>g</i>	<i>min</i>	
1 Fresh cell suspension			104
2 Supernatant after ultrasonic irradiation and 1st centrifugation	2,600	30	89
3 Supernatant of (2) after 2nd centrifugation	3,100	120	58
4 Sediment of (2) after 2nd centrifugation	3,100	120	98
5 Supernatant of (3) after 3rd centrifugation	100,000	23	25
6 Sediment of (3) after 3rd centrifugation	100,000	23	290

a lower hard packed layer of intact cells and (2) an upper gelatinous layer of ghost cells and debris.

The neutral, cell-free enzyme solution was stable for weeks at 0°. Stability decreased as the pH was lowered, below pH 4 the enzyme was rapidly inactivated. There was no loss in activity following either prolonged dialysis against neutral buffer solutions or precipitation with ammonium sulfate.

The L-amino acid oxidase is associated with small particles which can be readily separated from intact cells by differential centrifugation. In a gravitational field of $2600 \times g$, 1 hour sufficed to sediment quantitatively all bacterial cells, although little, if any, enzyme was sedimented from solution (cf Table III). In a gravitational field of $3100 \times g$ about 35 per cent of the enzyme was sedimented in 2 hours, whereas in a field of $100,000 \times g$ over 58 per cent of the enzyme was sedimented in 23 minutes. In the presence of phenylalanine as substrate, the QO₂ of the resuspended sediment obtained after high speed centrifugation was 290 in contrast to 104 for the

intact fresh cell and 89 for the cell-free juice after removal of the intact bacterial cells

Above 50°, the enzyme in neutral salt solution is unstable. Thus, 5 minutes at 55° destroyed 78 per cent of the activity, while 5 minutes at 60° destroyed all activity. The importance of efficient cooling during exposure to ultrasonic radiation is therefore obvious.

Enzyme activity was followed either manometrically by measuring the rate of oxygen uptake or colorimetrically in Thunberg tubes by following the decolorization time of methylene blue. The results obtained by these two methods were in good agreement. Apparently the rate of oxidation is

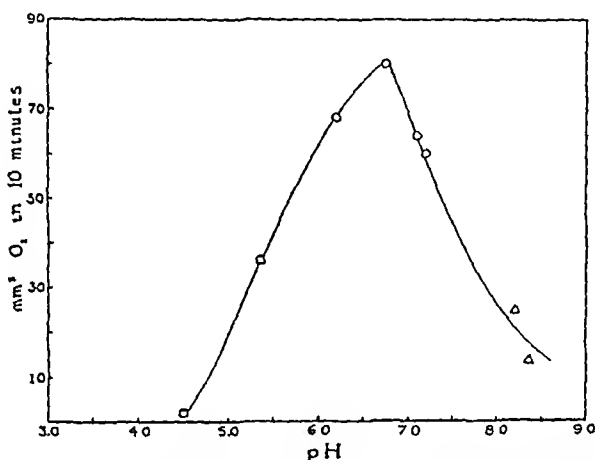


Fig. 1. Reaction velocity as a function of pH. Each manometer vessel contained 0.5 cc of enzyme, 1 cc of 0.1 M L-leucine, and 1.5 cc of 0.5 M buffer. Final volume 3 cc, alkali in center well, 38°. The following symbols indicate the nature of the buffer, □ acetate, ○ phosphate, and Δ dimethylglycine.

practically identical whether oxygen or methylene blue is used as hydrogen acceptor. In the manometric runs air was used in the gas space. Substitution of an atmosphere of pure oxygen for air had no influence on the rate of oxygen consumption. This indicated that the limiting reaction was not the rate of oxidation of the reduced enzyme by oxygen. Consistent with this interpretation was the fact that hydrogen acceptors like methylene blue did not increase the rate of oxygen uptake. Probably the limiting factor in the over-all reaction was the rate of reduction of the enzyme by the substrate.

Figs. 1 to 4 show the dependence of reaction velocity on pH, concentration of substrate, concentration of enzyme, and temperature respectively. The

optimum pH is 6.8. The molar concentration of substrate at which half the maximum velocity is reached is about 6×10^{-3} . Above 1.2×10^{-2} M, increase in the concentration of substrate does not lead to any change in the velocity of oxidation. Between $30-50^\circ$ there is a straight line relation between velocity and temperature. Above 50° enzyme destruction became

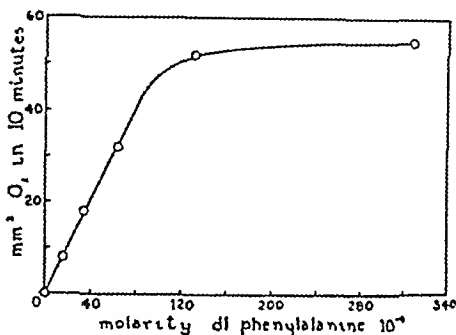


FIG 2 Reaction velocity as a function of substrate concentration. Each manometer vessel contained 0.5 cc of enzyme and 1 cc of 0.1 M phosphate buffer of pH 7.1. Final volume 3 cc, alkali in center well, 35° .

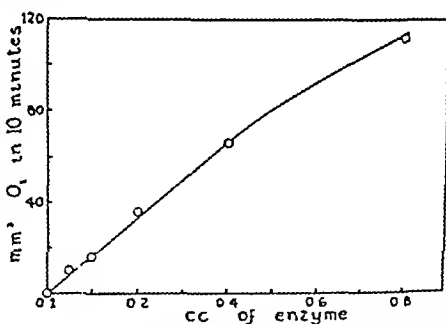


FIG 3 Reaction velocity as a function of enzyme concentration. Each manometer vessel contained 1 cc of 0.1 M L-leucine and 1 cc of 0.1 M phosphate buffer of pH 7.1. Final volume 3 cc, alkali in center well, 35° .

increasingly important. However, there is no simple explanation of the break in the curve below 30° . Over a restricted range of enzyme concentration, the rate of oxygen uptake is strictly proportional to the enzyme concentration. When the rate exceeds 100 cc mm³ of O₂ per 10 minutes, diffusion of oxygen from the gas space into the solution becomes a limiting factor under the conditions of the experiment and the linear relation no longer holds.

Inhibitors—Table IV summarizes the effects of various inhibitors on the activity of *l*-amino acid oxidase. Arsenite, fluoride, azide, iodoacetate, and sulfathiazole were without effect. 0.01 M HCN inhibited the enzyme 88

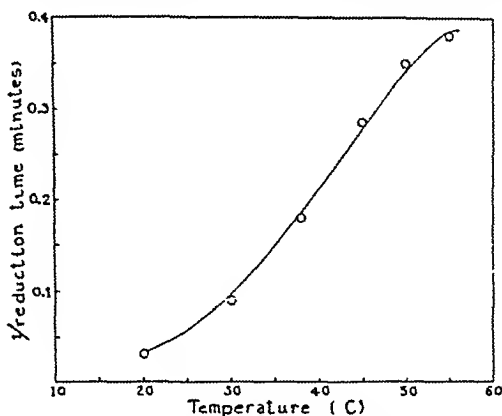


FIG 4 Reaction velocity as a function of temperature. Each Thunberg tube contained 1 cc. of 0.1 M *l*-leucine, 0.2 cc. of 0.1 per cent methylene blue, 1 cc. of enzyme, and 1 cc. of 0.05 M phosphate buffer of pH 7.1, 35°.

TABLE IV
Effect of Various Reagents on Activity of Enzyme

Reagent	Final concentration	Inhibition (aerobic conditions)
		per cent
Silver nitrate	M/1000	100
Cupric sulfate	M/1000	62
Mercuric chloride	M/1000	100
Sodium benzoate	M/100	0
" azide	M/100	0
" cyanide	M/300	76
" "	M/3000	22
" arsenite	M/1000	0
" fluoride	M/100	0
Sulfathiazole	M/100	0
Iodoacetic acid	M/100	0
Caprylic alcohol	Saturated solution	100

per cent when activity was estimated manometrically under aerobic conditions. However, no inhibition with this concentration of cyanide was observed when activity was estimated anaerobically in Thunberg tubes. This indicated that cyanide inhibited the oxidation of the reduced enzyme.

by molecular oxygen, and not the reduction of the enzyme by its substrate Caprylic alcohol, even in the minute traces in which it dissolves in water, completely inhibited the activity of the enzyme, both anaerobically and aerobically. Heavy metals like silver, copper, and mercury were also toxic in low concentrations. 0.01 M benzoic acid, which completely inhibits the oxidation of the *D*-amino acid oxidase (4) and glycine oxidase (5) of animal tissues, was without any effect on the *Proteus* enzyme. The *l*-amino acid oxidase of rat kidney was similarly insensitive to this reagent.

Distribution of Enzyme in Bacteria—Our criteria for the presence in other bacteria of an enzyme corresponding in specificity to the *l*-amino acid oxidase of *Proteus vulgaris* has been an increased rate of oxygen uptake in the presence of phenylalanine as substrate. The bacterial suspensions tested were thoroughly washed to reduce the blank uptake to negligible proportions. The equivalent of the *Proteus* enzyme was found in *Aerobacter aerogenes* and *Pseudomonas pyocyaneus*, but not in *Escherichia coli*, *Streptococcus hemolyticus*, *Diplococcus pneumoniae*, *Salmonella paratyphi*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Sarcina lutea*.

EXPERIMENTAL

Preparation of Cell Suspensions—*Proteus vulgaris* (Strain X-19) was grown in Roux bottles on meat infusion agar for 16 hours at 37°. The bacteria were harvested by washing off the surface growth with small portions of distilled water, and then filtering the washings through a double layer of muslin to remove agar particles. After hard packing in the centrifuge, the bacteria were resuspended and washed two times with distilled water. The final thick paste of cells was stored at 0° in 0.05 M phosphate buffer at pH 7.4. For manometric experiments, the stock suspension was diluted 1:50 with 0.05 M phosphate buffer of pH 7.4, and for exposure to ultrasonic irradiation it was diluted with an equal volume of distilled water.

Disintegration of Proteus vulgaris—Cell suspensions of *Proteus vulgaris* were rapidly disintegrated by exposure to ultrasound which was generated by a crystal-controlled oscillator operating at 1000 volts and putting out 500 watts. The piezoelectric quartz crystal used was 3 inches in diameter and was ground to a frequency of 600 kc. To prevent overheating of irradiated bacterial suspensions, the transformer oil which surrounded the crystal completely was circulated continuously by a centrifugal pump through copper coils in an ice-water mixture. The temperature of cell suspensions was never allowed to rise above 38°.

25 cc of a bacterial suspension containing 50 mg of dry weight of bacteria per cc were irradiated for 20 minutes in a 200 cc volumetric flask. The distance between the upper face of the quartz crystal and the bottom of the flask could be adjusted critically by a rack and pinion device to which the

flask was clamped. After irradiation, the suspension was spun down with hard packing in a conical head centrifuge over a period of 2 hours. The yellow, opalescent supernatant fluid was again centrifuged for an additional hour to insure that all cells were removed and was then stored at 0°.

Sources of Amino Acids—*L*-Leucine, *dl*-phenylalanine, *L*-proline, *L*-arginine, and *L*-histidine were products of Merck and Company. All the other amino acids used were obtained from the Eastman Kodak Company. We are indebted to Dr. S. Ratner for a sample of *dl*-N-methylleucine and to Dr. V. du Vigneaud for samples of *dl*-N-methylhistidine and *dl*-N-methylmethionine.

Method of Estimation—After the reaction mixtures were deproteinized by addition of freshly prepared metaphosphoric acid (final concentration 1.4 M) aliquots were taken for estimation of NH_3 and keto acids. Ammonia was driven over into boric acid by aeration at room temperature, according to the method of Sobel,² and then estimated by titration with $\sim 70\%$ HCl by use of the micro burette of Scholander *et al.* (6). Keto acids were estimated by the bisulfite method of Clift and Cook (7). Correction factors of 1.15 and 1.12 were employed for the keto acids of leucine and phenylalanine, respectively.

2,4-Dinitrophenylhydrazones of Keto Acids—50 cc. of a 2 per cent solution or suspension of the amino acid in 0.05 M phosphate buffer of pH 7.4 were mixed with an equal volume of bacterial suspension (stock suspension diluted 1:10). The mixture was aerated at 37° and the rate of the reaction followed manometrically in a pilot run. When no further oxygen uptake was observed, other than that due to the suspension without substrate, the mixture was centrifuged and the clear supernatant fluid mixed with $\frac{1}{2}$ volume of 6 N HCl. A 2 N HCl solution containing the theoretical quantity of 2,4-dinitrophenylhydrazine was then added. The hydrazones were recrystallized to constant melting point from mixtures of ethyl acetate and ligroin (*cf.* Table V).

The 2,4-dinitrophenylhydrazone of indolepyruvic acid rapidly resinified when exposed to acid at temperatures greater than 30°. Precaution was therefore taken to form and process the hydrazone at 0°. The hydrazones of the keto acids corresponding to leucine and tyrosine were separated from the hydrazones of the decarboxylation products of the keto acids by extracting the ethyl acetate solutions with 10 per cent sodium bicarbonate. This procedure left behind the non-acidic hydrazones in the ethyl acetate phase.

The hydrazones of the keto acids corresponding to arginine and histidine were insoluble in all common organic solvents, in water, and in 10 per cent sodium bicarbonate, but they were soluble in hot 3 N HCl. The hydrazones were dissolved in twice the minimum volume of hot 3 N HCl and allowed to

² Sobel, E., private communication.

crystallize out at 0° as the hydrated hydrochlorides. This procedure was repeated until a constant melting point had been reached. The hydrochlorides were converted to the free bases by heating at 140° for some 50 hours at 1 mm of Hg pressure. The crystalline structure of the hydrochlorides disappeared during the heating, and in both cases fine amorphous powders were obtained with a higher decomposition point.

Preparation of d-Amino Acids—From *dl*-amino acids, the *d* forms were prepared in good yield by the selective oxidation of the *l* forms. Samples of

TABLE V
Analyses of 2,4-Dinitrophenylhydrazones

2,4-Dinitrophenylhydrazone of	M p	Decomposition point	Calculated				Found			
			C	H	N	Cl	C	H	N	Cl
	°C	°C	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Phenylpyruvic acid	187*		52.31	3.60	16.26		52.40	3.94	15.84	
β Indolepyruvic acid	169		53.26	3.42	18.27		53.14	3.79	17.41	
<i>p</i> Hydroxyphenylpyruvic acid	175*		50.00	3.36	15.55		49.76	3.66	15.14	
α Keto γ methiobutyric acid	149*		40.24	3.68	17.07		40.25	3.73	16.63	
α Keto β methylvaleric acid	171*		46.46	4.55	18.10		46.41	4.59	17.97	
α Ketocaproic acid	134		46.46	4.55	18.10		46.67	4.60	18.11	
α Ketoisocaproic acid	155		46.46	4.55	18.10		46.47	4.77	17.68	
α Ketovaleric acid	160		44.58	4.08	18.92		44.67	4.09	18.63	
β -Imidazolepyruvic acid hydrochloride 2H ₂ O		192	35.4	3.72		8.73	35.8	3.90		8.65
β Imidazolepyruvic acid		239	43.12	3.02			42.74	2.87		
α Keto δ guanidovaleric acid hydrochloride 1H ₂ O		216	35.4	4.21		8.69	35.6	4.1		8.67
α Keto δ guanidovaleric acid		267	40.79	4.28			40.4	4.4		

* Cf (8)

pure *d*-methionine, *d*-leucine, and *d*-phenylalanine have been thus prepared. The following is a typical method of preparation.

100 cc of a 2 per cent solution of *dl*-methionine were mixed with 10 cc of a fresh stock suspension of *Proteus vulgaris* and the mixture was aerated for 5 hours at 37°, at which time the manometric pilot run showed no further oxygen uptake. The mixture was centrifuged and the clear supernatant fluid was evaporated to dryness *in vacuo*. The residue was taken up in 40 cc of water and centrifuged to remove insoluble material. The clear solution was warmed on a steam bath, and alcohol added to incipient turbidity.

After two recrystallizations, the yield was 0.5 gm, $[\alpha]_D^{20} = +7.5$, N 9.25 per cent. The sample of *D*-methionine was not attacked by the *L*-enzyme of *Proteus vulgaris* but was completely oxidized by the *D*-amino acid oxidase of pig kidney.

SUMMARY

Proteus vulgaris contains an enzyme which catalyzes the oxidative deamination of norleucine, phenylalanine, leucine, tryptophane, methionine, tyrosine, norvaline, histidine, arginine, isoleucine, and α -aminobutyric acid, the velocities being in the order named. For each molecule of amino acid oxidized, 1 atom of oxygen is taken up and 1 molecule of keto acid and ammonia formed. The enzyme is associated with insoluble particles which can be sedimented effectively only in gravitational fields higher than $3000 \times g$.

We are indebted to Miss Pauli for growing large quantities of *Proteus vulgaris*, to Dr D. Moore for carrying out ultracentrifugal runs, and to Mr William Saschek for the C, H, N analyses. This work has been supported by the John and Mary R. Markle Foundation, the Rockefeller Foundation, the Williams-Waterman Fund of the Research Corporation, the Nutrition Foundation, and the Lederle Laboratories. The piezoelectric quartz crystals for the ultrasonic apparatus were obtained through the generosity of the Federal Telephone and Radio Corporation and the August E. Miller Laboratories.

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SOME INTERRELATIONSHIPS IN GENERAL NITROGEN METABOLISM

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The feeding to rats of a small amount of amino acid in which the α -amino nitrogen is labeled with heavy nitrogen (N^{15}) results in the incorporation of about 50 per cent of the labeled nitrogen into the proteins of the animal tissues. A considerable proportion of the marked nitrogen in the proteins is still attached to the same species of carbon chain as existed in the compound fed. This is due to a direct introduction of the dietary amino acid into the protein by a process which involves the opening and closing of at least two peptide bonds. The amount of N^{15} thereby introduced depends on the speed of splitting and reformation of peptide bonds and on the concentration of N^{15} in the amino acid available for synthesis at the site of reaction (1).

The remainder (about two-thirds) of the N^{15} in the protein is found in the other amino acids and its presence there is due to transfer of nitrogen from the labeled amino acid to the other amino acid precursors, presumably keto acids. The amount of N^{15} introduced will depend on the rapidity of the processes, which involve transamination (2, 3), deamination, and reamination (4). The new amino acids, once formed, can be directly incorporated into the protein.

The concentration of N^{15} in a protein after the feeding of an isotopic amino acid has been taken (5) as a measure of the over-all chemical activity or rate of regeneration of the proteins of specific organs or tissues. Although there is no direct method for determining the dilution of the dietary amino acid by the same species of compound already present in the organism, it has been possible to conclude that the chemical activity of liver, plasma, and intestinal tract proteins is high as compared to that of muscle, skin, and connective tissue proteins (1). When leucine labeled with both deuterium and nitrogen was fed to rats for 3 days (1), at least 24 per cent of the leucine molecules originally present in the protein of the rat liver had been replaced by dietary leucine. The replacement would be half completed in about 7 days. In the same 3 day period more than one-third of this deposited leucine had lost its original nitrogen and had been regenerated with nitrogen from other sources. This experiment gives no indication as to the amount of leucine transferred to the liver proteins from other

tissue proteins Interpretation of such experiments, when carried out with non-essential amino acids, is further complicated by the ability of the organism to synthesize the carbon chain of the amino acid Introduction of these newly synthesized amino acids into the protein will tend to lower rather than to raise the isotope concentration

It does not at present seem feasible to determine the isotope concentration of the amino acids actually available for incorporation into protein at the site of the biological synthesis, but a technique previously utilized in this laboratory for the study of synthesis and degradation of creatine (6), blood proteins, and antibodies (7) is applicable A labeled compound is fed until adequate amounts are deposited, the rate at which the isotope then disappears from the tissue or tissues is a direct measure of metabolic activity

In order to obtain more information on the rates of incorporation of amino acids into proteins and the transfer of nitrogen from one carbon structure to another, a small proportion of isotopic glycine was incorporated in the diet of a number of rats for 3 days, the animals were killed in groups at intervals during the ensuing week, and the isotope concentration determined in specific tissues and certain of the amino acid components of their proteins From the data so obtained, it is possible to study the transfer of α -nitrogen among the amino acids of an organ, as well as the rate of transfer of nitrogen among the organs In order to secure supplementary information, it was considered desirable to study the process in tumor tissue as well as in normal tissue Rats inoculated with a transplantable Sarcoma R-39 were accordingly employed in this experiment

EXPERIMENTAL

Preparation of Glycine—Glycine was synthesized in the manner described by Schoenheimer and Ratner (8) It contained 34.4 atom per cent excess N^{15} Found,¹ N 19.1, theory, N 19.0

Feeding Experiment—Sixteen rats were inoculated with a transplanted Sarcoma R-39 under the skin of the rear thigh² 10 days after transplantation, ten rats were selected in which the tumors were not ulcerated and showed least signs of autolysis

These ten animals had an average weight of 122 gm (116 to 134 gm) They were given a diet of the following percentage composition: starch 83, yeast 5, salt mixture (9) 4, pure cottonseed oil (Wesson oil) 6, cod liver oil 2

¹ In these calculations the atomic weight of nitrogen is taken to be 14.34, since it contained 34.4 atom per cent excess N^{15}

² We are indebted to Dr. W. H. Woglom of the Department of Cancer Research of Columbia University both for preparing the transplants and for the valuable discussions on the results of these experiments

This diet contains 0.4 per cent N. As the rats consumed 9 gm of diet per day, they thus received 36 mg of unmarked nitrogen daily. After 2 days on this diet, each rat received in addition 146 mg of the isotopic glycine each day for 3 days.

After they had received the glycine for 3 days, three of the rats were killed (Group 0). The remaining seven rats were then kept on the stock diet. After 2 days, two of them were killed (Group 2). 2 days later (4 days after the rats had been put back on the stock diet) two other rats were killed (Group 4). The remaining three rats were killed 7 days after being

TABLE I
N¹⁵ Distribution in Proteins of Organs

Tissue	No. of rats	Group No	Protein		N ¹⁵ concentration in non protein N
			Total N	N ¹⁵ concentration	
			mg	atom per cent excess N ¹⁵	atom per cent excess N ¹⁵
Carcass	3	0	6710	0.217	0.509
"	2	2	4260	0.236	
"	2	4	4340	0.243	0.459
"	3	7	5400	0.278	0.383
Liver	3	0	375	1.34	2.48
"	2	2	202	1.19	1.75
"	2	4	200	1.03	1.52
"	3	7	257	0.857	1.38
Internal organs	3	0	385	0.860	1.89
" "	2	2	270	0.767	1.46
" "	2	4	257	0.696	0.926
" "	3	7	337	0.603	0.894
Tumor	3	0	357	1.13	2.79
"	2	2	250	1.22	2.37
"	2	4	202	1.15	1.88
"	3	7	315	1.03	1.68

put back on the stock diet (Group 7). The proteins of each group of animals were separated into four main fractions, (a) liver, (b) tumor, (c) internal organs which included spleen, intestinal tract, kidneys, and heart, and (d) the rest of the animal carcass which is principally muscle and skin. Each protein fraction was extracted thoroughly with 10 per cent trichloroacetic acid to remove non-protein N. The sixteen protein fractions were hydrolyzed with 20 per cent HCl. The isotope concentration of the proteins and the non-protein of the tissues of the liver, internal organs, carcass, and tumor are given in Table I.

Urinary Constituents—From the moment of addition of the labeled glycine, urine was collected. During the first 3 days, the rats were divided

into two groups containing three and seven rats. The urine of the three rats composing Group 0 was called Urine 0a, and that of the remaining seven rats was called Urine 0b. The urinary output of the seven rats during the succeeding days is called Urine 2. The urine output of the five remaining rats of the next 2 days was called Urine 4, and of the remaining three rats for the last 3 days, Urine 7. The nitrogen content and isotope concentration of the total urine, urea, and ammonia are given in Table II. No attempt was made to obtain a complete balance of the N^{15} administered. Ammonia was obtained from the urines by absorption with permittit and, from the filtrate, urea was isolated as dianthrydrylurea.

It is obvious that the isotope concentration of the urine must depend not only on the amount of the labeled amino acid metabolized but also on the total amount of nitrogen excreted. The isotope concentrations of

TABLE II
 N^{15} Concentration of Urinary Constituents

Period No	Urine No	No of rats	Period of collection	Total N excreted	N excreted per rat per day	N^{15} concentration of urine	N^{15} excreted per rat per day	N^{15} concentration of urea	N^{15} concentration of urinary NH_3
			days	m eq	m eq	atom per cent excess N^{15}	m eq	atom per cent excess N^{15}	atom per cent excess N^{15}
0	0a	3	3	24.2	2.69	2.16	0.0582	2.33	2.64
	0b	7	3	37.2	1.77	3.04	0.0539	3.28	3.67
2	2	7	2	28.8	2.06	2.18	0.0449	2.33	2.24
4	4	5	2	20.7	2.07	1.43	0.0296	1.35	1.36
7	7	3	3	15.0	1.67	1.12	0.0187	1.04	1.14

Urines 0a and 0b were markedly different, but the amount of N^{15} excreted daily per rat is almost the same in the two groups, namely, 0.058 and 0.054 miliequivalent of N^{15} .

As in previous experiments in which glycine was fed to rats, the concentration of N^{15} in the urea was slightly less than in the ammonia of the urine obtained during the feeding period. In the three periods after cessation of glycine feeding the ratio of the concentration of N^{15} in the ammonia to that of the urea drops below unity, then rises again. Despite the low nitrogen content of the diet (2.6 miliequivalents per day) the animals were in approximate nitrogen balance. The tumor has had no obvious effect on the isotope distribution (10) in the urinary constituents.

Isolation of Amino Acids—Amino acids were isolated from the protein hydrolysate by the usual procedures. Tyrosine was isolated by isoelectric precipitation. Glutamic acid was isolated by precipitation of the Ba salt in alcohol and reprecipitated as the hydrochloride. Histidine was isolated

as the mercuric complex (11) and purified as the 3,4-dichlorobenzenesulfonate (12)

Glycine was isolated as the trioxalatochromate by the method of Bergmann *et al* (13) and purified as the *p*-toluenesulfonyl derivative Arginine was isolated as the flavianate and purified either as the monohydrochloride or as the *p*-toluenesulfonyl derivative

TABLE III
Analytical Constants of Isolated Amino Acids

Tissue	Group No	p Toluenesulfonyl glycine		Arginine HCl	p Toluene-sulfonyl arginine	Glutamic HCl	Tyrosine	3,4 Dichloro-benzene-disulfonate of histidine
			M p					
		per cent N	C					
Carcass	0	6 12	147-148	26 5		7 53	7 78	6 92
"	2	6 12	147-148		16 8	7 67	7 74	6 89
"	4	6 16	147-148		17 0	7 63		7 06
"	7	6 11	146-147	26 6		7 49	7 67	6 66
Liver	0	6 06	147-148		16 9	7 67	7 74	
"	2			*		7 70	7 74	
"	4			*				
"	7	5 8	147		16 7	7 69	7 54	
Internal organs	0	6 12	147-148		16 9	7 71	7 73	
" "	2	6 13	148	*		7 65		
" "	4	6 12	147	*			7 75	
" "	7	6 12	146-147		17 0	7 60	7 70	
Tumor	0	6 20	146-147		16 9	7 64	7 77	
"	2			*		7 60†	7 65	
"	4			*		7 75		
"	7	6 12	147		17 0	7 62†	7 80	
Theory		6 1		26 6	17 0	7 63	7 74	6 88

* Flavianate decomposed and analyzed

† $[\alpha]_D = 31.8^\circ$ (calculated for glutamic acid)

Protein hydrolysates were made alkaline with $\text{Ba}(\text{OH})_2$ and ammonia collected by aeration This is regarded as amide nitrogen

The nitrogen determination of all amino acids isolated and the melting points of derivatives, when these were made, are given in Table III The isotope concentrations of the proteins and of the amino acids of the proteins are plotted in Figs 1 to 5

Distribution of N^{15} in Arginine—To study the distribution of N^{15} in the arginine, either the *p*-toluenesulfonylarginine or arginine monohydrochloride was refluxed with saturated $\text{Ba}(\text{OH})_2$ solution for 24 hours and the liberated ammonia swept into a dilute sulfuric acid trap by a stream of

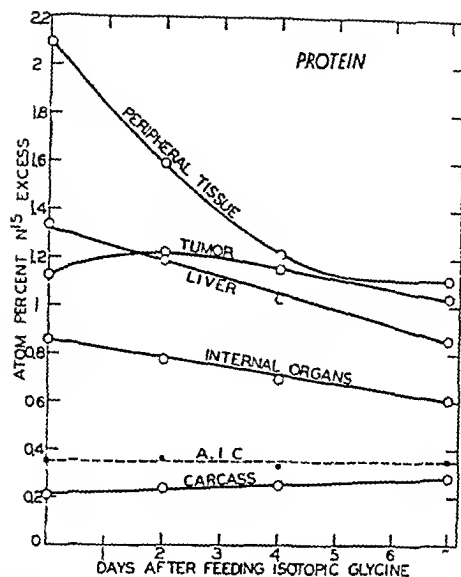


FIG 1 Concentration of N^{15} in tissue proteins after feeding isotopic glycine
A I C represents the average isotope concentration

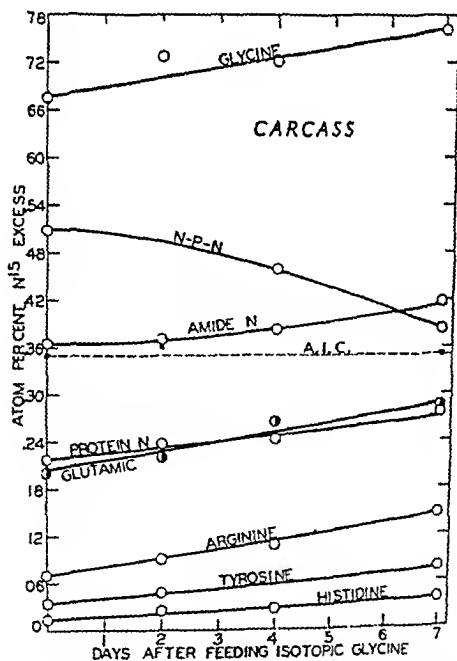


FIG 2 Concentration of N^{15} in carcass constituents after feeding isotopic glycine

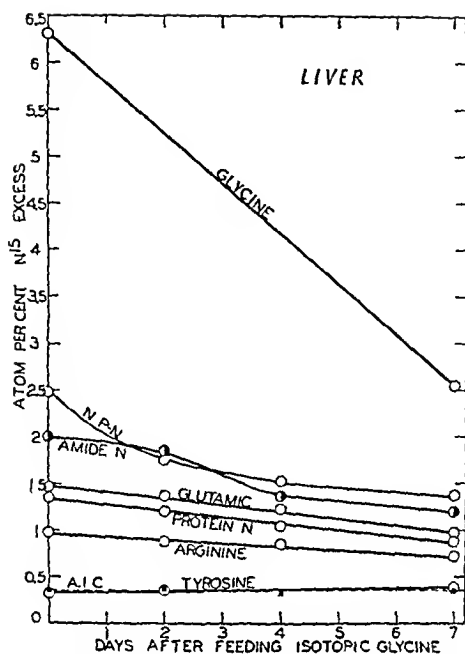


FIG 3 Concentration of N^{15} in liver constituents after feeding isotopic glycine

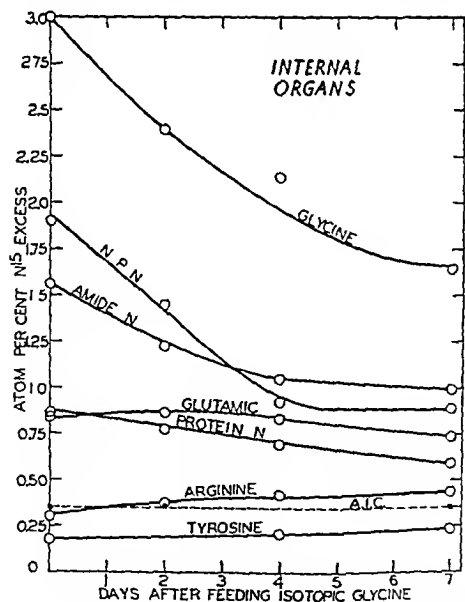


FIG 4 Concentration of N^{15} in internal organ constituents after feeding isotopic glycine

nitrogen The ornithine was isolated either as the α -p toluenesulfonyl derivative or as the dibenzoyl derivative (Table IV)

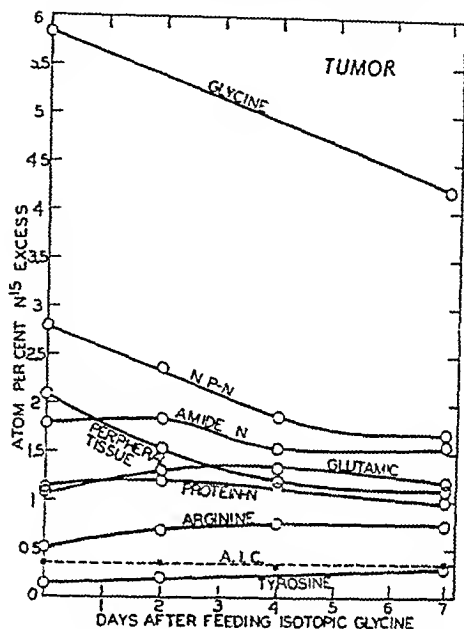


FIG 5 Concentration of N^{15} in tumor constituents after feeding isotopic glycine

TABLE IV

Concentration of N^{15} in Amidine and Ornithine Nitrogen of Arginine

The values are given in atom per cent excess N^{15}

Tissue	Group No	N^{15} concentrations in			
		Arginine	Amidine N	Ornithine found	Ornithine* calculated
Carcass	0	0.070	0.106	0.031	0.034
"	2	0.090	0.112		0.068
"	4	0.109	0.157	0.063	0.061
"	7	0.147	0.204	0.087	0.090
Liver	0	0.967	1.86		0.07
"	4	0.854	1.28		0.43

* These values are calculated from the isotope concentrations of the arginine and the amidine nitrogen $C_{\text{ornithine}} = 2C_{\text{arginine}} - C_{\text{amidine}}$

Distribution of N^{15} in Ornithine—To 0.5 gm of α -toluenesulfonyl ornithine (prepared from the arginine of carcass from Group 4) in 10 cc of water, 10

cc of a 30 per cent solution of barium nitrite and 5 cc of glacial acetic acid were added. After 24 hours at room temperature the reaction mixture was extracted continuously with ether for 8 hours. The ether was evaporated off from the ethereal extract and the residue freed of acetic acid by evaporation in a vacuum desiccator over sodium hydroxide. The residue was dissolved in alcohol and precipitated as an oil by the addition of water. The dry oily material behaved as a lactone on titration with standard alkali. The equivalent weight determined by back titration after the material was dissolved in a slight excess of warm alkali was found to be 270, calculated for the lactone of α -toluenesulfonamino- δ -hydroxyvaleric acid ($C_{12}H_{15}O_4NS$) 269. Calculated, N 5.2, found, N 5.0.

As the lactone could not be crystallized, it was converted into the amide of α -toluenesulfonamino- δ -hydroxyvaleric acid by treatment with 50 cc of concentrated aqueous ammonia or 50 cc of saturated ammoniacal alcohol solution. The solution was kept at 0° for 24 to 48 hours. The solution was then taken to dryness *in vacuo* on a water bath kept at 40–50°. The residue was recrystallized several times from alcohol. Yield 50 mg, m p 182–183° with decomposition. The melting point was markedly depressed when the material was mixed with α -toluenesulfonylornithine.

$C_{12}H_{15}O_4N_2S$	Calculated	C 50.31, H 6.34, N 9.78, amide N 4.89
	Found	" 50.33, " 6.51, " 9.74, " " 4.85

The N^{15} concentration was 0.032 atom per cent excess.

No attempt was made actually to determine the position of the hydroxy group in the above compound. It is well known that in some cases treatment of terminal amino groups with nitrous acid may result in a shift giving a secondary rather than the expected primary alcohol. It is immaterial for the determination of the distribution of N^{15} in ornithine whether a secondary or a primary alcohol was formed.

An exploratory experiment was carried out to determine whether the tumor had any marked effect on the nitrogen turnover of the liver protein. Two normal rats (225 and 231 gm) were kept on the same diet as in the above experiment. To this basal diet were added 25 mg of glycine per 100 gm of rat (N^{15} concentration, 33.7 atom per cent N^{15} excess) for 3 days. One rat (Rat A) was killed 24 hours after the last addition of labeled glycine. The other rat (Rat B) was kept for 10 days on the basal diet and then sacrificed. The liver and carcass protein hydrolysates prepared as in the above experiment were analyzed for N^{15} . The atom per cent N^{15} excess found in Rat A was 0.548 for the liver and 0.079 for the carcass, for Rat B, 0.250 and 0.079 respectively. This experiment indicates that the turnover rate was not different in these animals from those which had tumors.

DISCUSSION

Proteins—From the sums of the numbers of equivalents of N^{15} and of total nitrogen respectively in the proteins of the entire animal (see Table I) the general average N^{15} concentration (or average isotope concentration) can be calculated. These values are 0.35, 0.36, 0.33, and 0.35 atom per cent N^{15} excess for Groups 0, 2, 4, and 7 respectively. The non-protein N was not included in this calculation, as a large part of it is urea and is not available for synthetic reactions. As after cessation of the feeding of labeled glycine the normal nitrogen intake, 36 mg per rat per day, was small in comparison to the total nitrogen content of a rat, the average isotope concentration for the animal as a whole should remain almost constant. In every tissue protein the isotope concentration should theoretically approach this value with time, from either direction.

In agreement with this prediction, the isotope concentration of the carcass proteins, which are lower than the average during the experimental period, was found to increase from Group 0 through Group 7. With the exception of the total tumor protein, the other proteins, which start above the average isotope concentration, steadily decrease with time (Fig 1). The total tumor protein initially increases in isotope concentration, then slowly decreases. The isotope concentrations of the non-protein N in the different organs from any particular group of animals differ considerably. This is notable, as the nitrogenous constituents of the non-protein N are freely diffusible and should rapidly equilibrate. For ready comparison the isotope concentrations of N^{15} in the proteins of the various tissues are plotted in Fig 1, in which is also drawn the average isotope concentration for the several groups of animals. These values are remarkably constant, indicating that in each group of animals very nearly the same amount of labeled glycine nitrogen has been deposited per gm of total nitrogen. Had the experiment continued for a sufficiently long period, all the nitrogen which interacts with the dietary glycine nitrogen should have reached this concentration. After the inclusion of labeled glycine in the diet had been discontinued, all the metabolically active compounds having an isotope concentration higher than the general average should tend to decrease in isotope concentration, all metabolically active compounds having a lower concentration of N^{15} should tend to increase in isotope concentration, approaching the average value. Of course the nitrogen compounds which do not interact with the dietary nitrogen, such as lysine, will not increase in isotope content, and compounds containing 2 or more nitrogen atoms, of which some do not interchange, will approach a level proportionally lower than the average.

The isotope concentrations in Group 0 are similar to those found in the previous glycine experiment (10). The nitrogen of the carcass proteins

has an isotope concentration of about one-seventh that of liver, though, because it has a much larger total nitrogen content, the carcass has accepted more glycine nitrogen. As can be expected, the nitrogen of the liver proteins, which initially has an isotope concentration above the average, declines in concentration as the experiment proceeds. If, as we assume, the liver protein will eventually reach the average value, then, when the liver protein reaches a concentration of 0.84 atom per cent excess N^{15} (half way from 1.34 to the average of 0.35 per cent), approximately half of the labeled nitrogen will have been transferred to the other organs and in turn will have been replaced by nitrogen of the other organs. The time for this was estimated to be about 7 days. This calculation is not exact, for the concentration of isotope in the nitrogen available to the liver may not be the general average for the proteins of the rat, though it must be quite close to it. On the basic assumption that the labeled nitrogen is treated no differently from the normal nitrogen, the time during which the N^{15} concentration drops to 0.84 per cent will also be the time necessary for half the nitrogen of the liver to be regenerated. It represents a resultant of the various rates of transfer of all the amino acids of the protein.

The half life time of the livers of these animals seems in no detectable way to be influenced by the presence of a sarcoma in the animal. For, from the data obtained on the livers of the normal animals (see above) the half life time of the liver proteins can be calculated. This was found to be 6.5 days. This calculation also took into account the fact that the excess N^{15} concentration of the liver was approaching, not zero, but the general average excess N^{15} concentration, which was estimated to be 0.09 atom per cent N^{15} excess, slightly higher than the N^{15} concentration of the carcass observed at the end of the experiments.

The rise in isotope concentration of the carcass proteins is the result of the transfer of isotopically rich nitrogen to the carcass from other organs. The values for the internal organs represent the analysis of a mixture of tissues, all of which may have different mean rates of nitrogen metabolism. Since they fall between those for the liver and those for the carcass, the slope of the N^{15} concentration curve is not simply related to the half time of nitrogen regeneration but will depend on the relative rates of nitrogen transfer from the liver and the carcass to these tissues. It is obvious that if the internal organ-carcass nitrogen interactions were very slow and the internal organ-liver interaction fast, at least during the first 6 days, a regeneration could be indicated by a rising N^{15} concentration. The actual slope of the curve will depend on the rates of these two interactions as well as the regeneration rate.

It seems plausible, however, that the internal organs are receiving their nitrogen principally from the carcass, since the N^{15} level is dropping. We

can, by a calculation similar to that used in the case of the liver, estimate the half time of regeneration (time necessary to reach 0.60 per cent) to be about 7 days. Any transfer of nitrogen from the liver to the internal organs will tend to decrease the slope of the curve showing the relation of concentration to time for the internal organs and would thus yield a half time longer than the true one. The estimate of the half time of the liver protein is in turn subject to similar considerations, for, from the 2nd to the 7th day, the tumor protein has a higher concentration than the liver. It is doubtful that these considerations are quantitatively important.

Liver—The isotope concentrations in all the constituents which we have isolated from the livers of the four groups, with the exception of tyrosine, decrease with time.

At the end of the 3 day period in which labeled glycine was fed (Fig. 3), 18 per cent ($6.3 \times 100/34.4$) of the glycine nitrogen of liver protein had been replaced by dietary glycine nitrogen. Slightly over 6 per cent of the liver glycine has been replaced each day in the protein by dietary glycine. This rate of replacement is a minimum value, since in the calculation we assume that the dietary glycine fed is available to the cell undiluted by preformed glycine of the tissues or by newly synthesized glycine, neither of which presumably contains much isotope. The actual rate of replacement must be higher than 6 per cent per day. In a previous experiment, in which the glycine supplement was one-half of that in this experiment, a minimum figure of 3 per cent per day was found (10). From the slope of the glycine curve in Fig. 3, the rate of glycine replacement can be calculated to be about 10 per cent per day at a minimum, equivalent to a half time of 5 to 6 days. This value is nearly the same as the half time for the transfer of nitrogen from liver to other proteins.

In this calculation we assume that the isotope concentration in the glycine replacing the liver glycine is 0.70 atom per cent excess N^{15} , i.e., the average isotope concentration in the carcass protein glycine. If the amino acid composition of the liver is not a function of the diet, then on an adequate nitrogen diet the rate of replacement of glycine in the liver protein should not increase by more than a factor of 2, since it is unlikely that the concentration of free glycine can increase greatly by the feeding of protein. A large influx of free glycine would be expected to be rapidly oxidatively deaminized. We therefore conclude that in the normal rat liver at least 10 per cent of the glycine is replaced by glycine of other organs or of the diet. This figure compares with a replacement rate of 8 per cent per day found for leucine in rat liver (1). These values illustrate the remarkable chemical instability of the cell structures.

We cannot determine from these data whether the rate-determining step is the splitting and reformation of the peptide links or the nitrogen transfer from glycine to other amino acids.

It will be noted that tyrosine, which initially has an isotope concentration of 0.32 atom per cent excess N^{15} , rises until, on the 7th day, it reaches a concentration of 0.38 atom per cent excess N^{15} , which is higher than the average. As the isotope concentration in the liver tyrosine is greater than the tyrosine of the other tissues, this shows that the rate of transfer of nitrogen from the other amino acids of the liver having a higher isotope concentration to tyrosine is faster than the interchange of tyrosine of liver and other organs. The chemical processes concerned with the redistribution of α -nitrogen in the liver seem to take place more rapidly than the liberation of intact amino acids to other tissues.

The isotope concentration found in the glutamic acid is, during the entire experimental period, nearly the same as that of the total protein nitrogen which represents the average N^{15} concentration of the amino acids. This is true for the other proteins investigated and is in accord with the current view that glutamic acid occupies a central and very active part in the nitrogen transfer reactions.

Both the amide nitrogen and the non-protein N have isotope concentrations above that of the protein nitrogen. As the composition of the non-protein N is not well known, no conclusions can be drawn from this fact. On the other hand, the ammonia available for formation of amide groups in the tissue proteins appears to have an isotope concentration considerably above that of the average protein nitrogen. The isotope level in the amide N will be discussed further with regard to urea formation.

Urine—As in the previous glycine experiment (10), the initial ammonia isotope concentration is above that of the urea. In the later periods these values approach each other (Fig. 6). During the entire experimental period, however, the isotope concentration in the urea is above that of any protein of the animal body and above that of any nitrogenous sample other than liver glycine. According to the Krebs and Henseleit "arginine cycle" theory (14), the isotope concentration of the urea formed at any moment should be the same as that of the "free arginine" of the liver. The relation of free arginine to protein arginine has been discussed in a previous publication (15). The concentration of N^{15} in the urea is much higher than that in the liver arginine at any period. Sufficient arginine was isolated from the livers of Groups 0 and 4, as well as from the carcass proteins of Groups 0, 2, 4, and 7 to permit degradation and isotope analysis of the ornithine and ammonia. These values are given in Table IV. As we did not in all cases obtain sufficient ornithine for N^{15} analysis, we have calculated the N^{15} concentration of the ornithine from those of the arginine and the amidine group. In those cases in which the ornithine was analyzed, excellent agreement with the calculated values was found.

The isotope concentration of the urea excreted during the 3 day preliminary feeding period by the three rats which were killed at the end of that

period is 2.33 atom per cent excess (see Table II), while the N^{15} concentration in the amidine group of the liver arginine of those animals is 1.86 atom per cent N^{15} excess (see Table IV). It is not surprising that these two values do not exactly agree, since one of them is a 3 day average while the other is a value at one instant. At the beginning of the experimental period, the isotope concentration of the amidine group of the protein arginine was zero and must have risen to the observed value, 1.86 per cent. The amidine group of the free arginine, however, must have very rapidly taken up isotopic nitrogen liberated from the dietary glycine, for the rate of

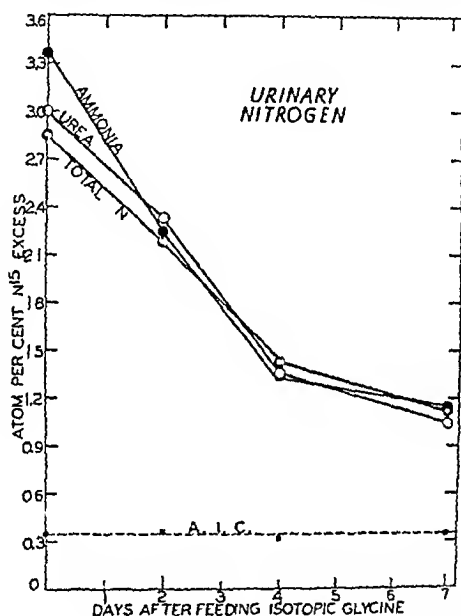


FIG. 6 Concentration of N^{15} in urinary constituents after feeding isotopic glycine

conversion of ammonia to urea is very rapid compared to the rate of introduction of labeled nitrogen into the protein.

In the animals sacrificed 4 days after the cessation of glycine feeding, the two values are apparently in much better agreement. Here also the urea value is the average of the output for the 3rd and 4th days. We have no direct information as to the average isotope concentration in the amidine group of the liver arginine for this period, but it must certainly have been higher than 1.28 atom per cent excess. The isotope concentration in this grouping was 1.86 per cent in Group 0. If the change of concentration be assumed to be linear with time, the N^{15} concentration in Group 2 can be

estimated as 1.57 per cent and the average value of the amidine group for the 3rd and 4th days about 1.43 per cent N^{15} . This rough value is in excellent agreement with the concentration found in the urea, 1.35 atom per cent excess N^{15} . We would expect that at this period sufficient time had elapsed to permit the free arginine and protein arginine to attain the same isotope concentration.

When the isotope concentration in the amidine of the carcass arginine was determined, it was found in all cases to be much lower than that of the urinary urea, in agreement with the view that the chief site of urea formation is the liver.

The isotope concentrations in the liver amide nitrogen for Groups 0 and 4 are very nearly the same as those of the amidine group of the liver arginine. Similar results (amidine N, 0.069 per cent, amide N, 0.065 per cent) were found in the previous glycine experiment (10). We cannot conclude from these data that they support the theory of Leuthardt (16) that the amide nitrogen of glutamine is the source of the nitrogen of the amidine group of arginine, for these figures may be merely a reflection of the fact that both the amidine group and the amide groups are formed from ammonia. However, when *l*-leucine was fed (1), the amidine nitrogen had a considerably higher isotope concentration than the amide N (0.079 and 0.051 respectively). In the experiment in which ammonium citrate was fed (17), the liver arginine had a higher concentration than the amide nitrogen. This finding is consistent with the view that ammonia is directly employed in the arginine cycle.

During the 7 days after cessation of labeled glycine feeding, Group 7 excreted about 0.205 miliequivalent of N^{15} per rat. The total N^{15} content of a rat liver at zero time in Group 0 was about 0.120 miliequivalent of N^{15} , and at the 7th day 0.053 miliequivalent of N^{15} . Each liver thus lost about 0.067 miliequivalent of N^{15} during the 7 days but had excreted in the urine, principally as urea, 0.205 miliequivalent of N^{15} . Other organs must have supplied N^{15} for excretion. We are, however, faced with the difficulty that during the entire period the isotope concentration of the urinary urea is higher than that of any organ, with the exception of the tumor on the 7th day. If, for example, the carcass were supplying for excretion nitrogen of its own isotope concentration (0.25 atom per cent excess N^{15}), then the excreted nitrogen would be expected to have the same low concentration.

There can be little doubt that the source of the major part of the urinary nitrogen is actually the carcass. The fact that its average isotope concentration is much lower than that of the excreted nitrogen can plausibly be explained by the hypothesis that the tissue we have called carcass is not only histologically heterogeneous but is also metabolically heterogeneous, at least as regards rates of reaction. For example, the muscle proteins may

consist of a small part of metabolically active and a large part of relatively inert components. This active protein could be the source of the high concentration N^{15} employed by the liver for urea formation.³

Internal Organs—The interpretation of the data derived from analysis of the internal organs is complicated by the heterogeneity of the tissues. The findings, however, represent the average behavior of metabolically active proteins and resemble the results found for the liver. It is of interest that, as mentioned previously, the isotope concentrations in non-protein N and in amide N are quite different from those of the liver. The concentration of N^{15} in the glycine falls with a half time of about 7 days (Fig. 4).

Carcass—As with the other proteins, the concentration of N^{15} in the glutamic acid is very nearly the same as that in the total protein (Fig. 2). The rapidity with which it comes to equilibrium with the average N^{15} content of all the other amino acids emphasizes the central rôle which is played by this amino acid (1, 2, 4).

The isotope content of the tissue proteins of the carcass of Group 0 and their component amino acids, histidine, glutamic acid, tyrosine, and arginine, is lower than the average value (0.35 per cent). The curves of these proteins and amino acids have, therefore, a positive slope and rise toward the average isotope concentration (Fig. 2). The N^{15} content of the glycine of Group 0 carcass was 0.68 per cent, twice as high as the average, and became still higher in the later groups (Figs. 2 and 7). It is obvious that the glycine of the carcass is not the immediate or only source of the nitrogen transferred to the other carcass amino acids, for, if it were, its isotope concentration would fall. The glycine, as well as the other amino acids analyzed, must have either interacted or been replaced by amino acids of a higher isotope concentration. *It appears that in the carcass the nitrogen transfer between glycine and the other amino acids is overshadowed by the process of incorporation of residues of intact glycine originating in other tissues.* Liver glycine, transported by the blood as non-protein nitrogen or in blood proteins, enters into the muscle proteins faster than the muscle proteins can redistribute its nitrogen, the general transamination rate must be relatively slow in the carcass.

Tumor Protein—The interpretation of the isotope concentration of the tumor proteins is complicated by the rapid growth as well as some necrosis of the central portions of the tumor. Small samples (~2 mg. of N) were therefore taken of the peripheral tissue and N^{15} concentrations of its protein directly determined. The values obtained are shown in Fig. 1. The remaining tissue was worked up as were the other proteins. The high N^{15} concentration found in the peripheral tumor protein of Group 0 indicates

³ In a test experiment we have been able to fractionate the proteins of muscle into fractions having widely different isotope concentrations.

the rapid metabolism of this protein as is also shown by the subsequent rapid decline. The tumor as a whole, however, seems to be slightly less active than liver protein, but, in marked contrast, it increased in N^{15} concentration for 2 days after the feeding of labeled glycine was discontinued. This increase continued until the concentration in liver and tumor became equal, then a slow decrease set in. It must be emphasized that, in spite of the central necrosis, the isotope concentrations measured were of protein and not of non-protein N which had been removed by

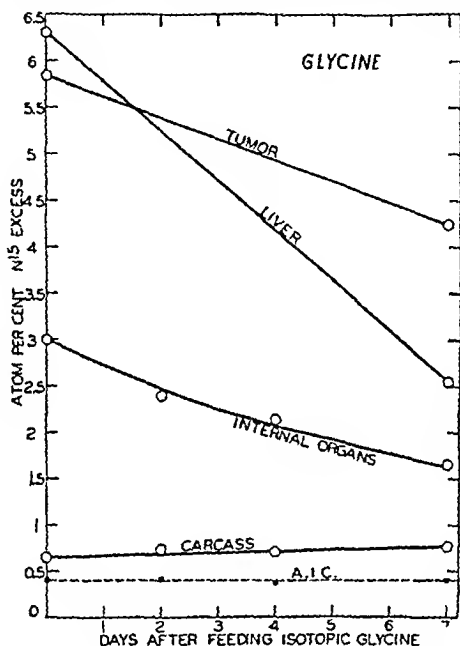


FIG 7 Concentration of N^{15} in glycine of various tissues after feeding isotopic glycine

trichloroacetic acid extraction. The total tumor protein accepts nitrogen almost as rapidly as liver but loses it by transfer to other proteins at a rate of about one-half that of liver. (The tumor in the last 5 days of the experiment decreased from 1.20 to 1.03 atom per cent excess N^{15} , while the liver decreased from 1.20 to 0.86.)

The slow fall of isotope concentration in tumor protein may be merely a reflection of the poor blood supply to the inner (in contrast to the outer) regions of the tumor. The peripheral protein of the tumor shows an initial value twice that of the liver. The decline in both the peripheral

tumor protein and the whole tumor cannot be interpreted simply as being caused by regeneration, for these tissues were growing. Any growth in which nitrogen of a lower isotope concentration than that of the tumor nitrogen were employed would result in a decline which would be more pronounced the more rapid the growth.

The rapidly growing sarcoma, which arises from the metabolically sluggish connective tissue, obviously must have a much higher over-all proteolytic activity than its parent tissue. The mass of a tissue is resultant of two processes, synthetic and degradative. Growth may be the result of a retarded degradative rate or of an accelerated synthetic rate, or of both. The rates of these processes must be determined by two major factors, the concentration of effective enzymes and the concentration of the substrate. There is at present no obvious way in which these factors can be evaluated.

Because of experimental difficulties glycine could be isolated only from the proteins of Groups 0 and 7. The regularity of the curves obtained for the other nitrogenous constituents of the protein suggest that the points for Groups 2 and 4 would probably lie close to the straight line drawn through the two points (Fig 5). The slope of this curve is strikingly less than that found for glycine in liver and internal organs. The half time for decline of the isotope concentration in tumor glycines was approximately 12 days. For ready comparison, we have plotted in Fig 7 the isotope concentration of the glycine in the various tissues. The N^{15} concentration in the tumor glycine in Group 0 was almost as high as that of the liver glycine. Though this tissue had rapidly accepted the labeled amino acid, it parted with it far less rapidly. The actual rate of loss of labeled glycine from the protein must have been slower than is indicated by the slope of the curve, for, in contradistinction to all other tissues, the tumor was growing and contained more glycine at the end of the experimental period than it did at the beginning.

The isotope concentrations of all the other amino acids isolated from tumors increase during the experimental period (Fig 5).

Arginine—The isotope concentrations in the arginine of the various proteins are shown in Fig 8. In every organ except the liver they increase during the experimental period. The interpretation of these changes is complicated by the fact that arginine contains 4 nitrogen atoms. The 2 nitrogen atoms of the amidine group are identical, those of the ornithine moiety may interact with the labeled glycine at different rates. The rise in isotope concentration of the carcass arginine must be due to an isotope increase in one or more of the three types of nitrogen. The figures in Table IV show such an increase in all three. That in the nitrogen of the ornithine correlates with the fact that arginine can be synthesized slowly by the rat (18). In order to investigate the distribution of the N^{15} in the α - and

δ -amino groups of ornithine, a sample of ornithine containing 0.063 atom per cent excess N^{15} obtained from arginine of the carcass of Group 4 was converted to α -toluenesulfonamino- δ -hydroxyvaleric acid amide. This compound contained 0.032 atom per cent excess N^{15} . As the amide was made with normal ammonia, the isotope concentration of the α -amino group of this compound was 0.064 atom per cent excess N^{15} . Since the isotope concentration of the ornithine N was 0.063 atom per cent, the δ -amino nitrogen must have been almost the same. Whatever the mechanism for the formation of ornithine may be, it is clear that both the α - and

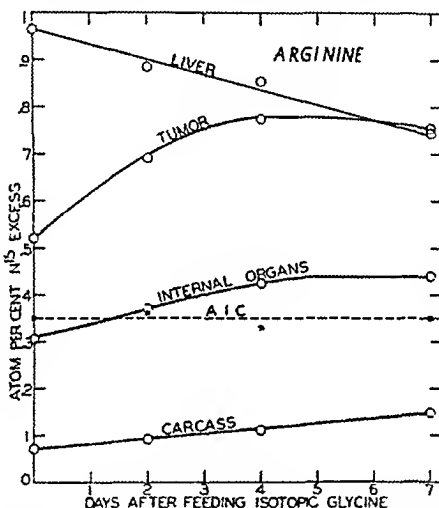


FIG. 8. Concentration of N^{15} in arginine of various tissues after feeding isotopic glycine.

δ -amino groups can obtain their nitrogen from dietary nitrogen. Further investigation will be necessary to determine whether the equality of isotope concentrations is of biological significance or merely fortuitous.

SUMMARY

From the data obtained from isotopic analysis of the proteins of rats killed at intervals of 0, 2, 4, and 7 days after they had been fed glycine labeled with N^{15} , the following conclusions may be drawn:

1. Half of the total nitrogen of the liver protein is replaced by nitrogen of the other proteins and the diet in 7 days. The value of this half time does not appear to be influenced by the presence of a transplanted sarcoma in the body.

2 About 10 per cent of the liver glycine nitrogen is replaced daily by glycine from other sources

3 The carcass proteins incorporate glycine from other organs faster than they transfer glycine nitrogen to other amino acids

4 The concentration of N^{15} in urinary urea is very nearly the same as that of the amidine group of arginine of liver protein

5 The muscle appears to be composed of proteins which are metabolically heterogeneous, some must interact slowly with dietary nitrogen, and others very rapidly

6 The protein of the tumor which was investigated incorporates dietary nitrogen almost as rapidly as liver protein, but releases it more slowly. The relationship of regeneration to growth is discussed

7 The non-protein N fractions of the various tissues have different isotope concentrations. Even though the components of the non-protein N are freely diffusible, each organ has its own characteristic composition

8 The preparation of α -toluenesulfonamino- δ -hydroxyvaleric acid amide from ornithine is described. Analysis of this compound shows that the synthesis of ornithine in the rat takes place in such manner that dietary nitrogen is incorporated in both the α - and δ -amino groups

9 The interrelationships of nitrogen transport among the organs are

The authors wish to express their appreciation to Mr I. Sucher for the isotope analyses

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FURTHER STUDIES ON VITAMINS B₁₀ AND B₁₁ AND THEIR RELATION TO "FOLIC ACID" ACTIVITY*

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A previous report from this laboratory (1) demonstrated the existence of two water-soluble vitamins needed by the chick, namely, vitamin B₁₀, essential for proper feather formation, and vitamin B₁₁, necessary for growth. Both of these vitamins, present in a liver concentrate (Super Filtrol eluate), were shown to be distinct from "folic acid"¹ which was also present in the liver concentrate. All three of these factors were adsorbed and eluted from Norit and Super Filtrol but were partially separated by fractional precipitation with ethanol.

This paper presents further progress toward the separation and isolation of vitamins B₁₀ and B₁₁ and gives additional information on their chemical properties as they exist in impure concentrates. The relationship of these substances to "folic acid" is pointed out.

EXPERIMENTAL

To study the distribution and properties of vitamins B₁₀ and B₁₁ all liver preparations were assayed for feathering and growth activity with white Leghorn chicks (in groups of six) over a 4 weeks period. The experimental conditions and the basal ration, No. 486K, have been reported previously (1, 5). This ration consists essentially of dextrin, alcohol-extracted casein, gelatin, soy bean oil, salts, cystine, and ample levels of known crystalline fat- and water-soluble vitamins. The level of biotin has been raised from

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¹ The term "folic acid" has been used in this paper to designate any substance necessary for the growth of *Streptococcus lactis* R and *Lactobacillus casei* when grown on a defined medium (2, 3). Mitchell, Snell, and Williams (4) first used this term for "an acid nutritive" which had been obtained nearly pure and which was necessary for the growth of *Streptococcus lactis* R. They reported that the substance stimulated the growth of *Lactobacillus casei* under similar conditions.

15 to 20 γ per 100 gm of ration Normal growth and feather formation are not attained by chicks fed this basal ration unless a source of the unknown vitamins is supplied

Assays were made by incorporating each preparation uniformly in the basal ration and the response obtained compared to that of chicks receiving the basal ration with and without solubilized liver A response in feathering, as determined by the use of the scale in Fig 1, indicated the presence of vitamin B₁₀, while vitamin B₁₁ was measured by an increase in growth

"Folic acid" assays were performed either by the method of Mitchell and Snell (2) with *Streptococcus lactis* R as the test organism and solubilized

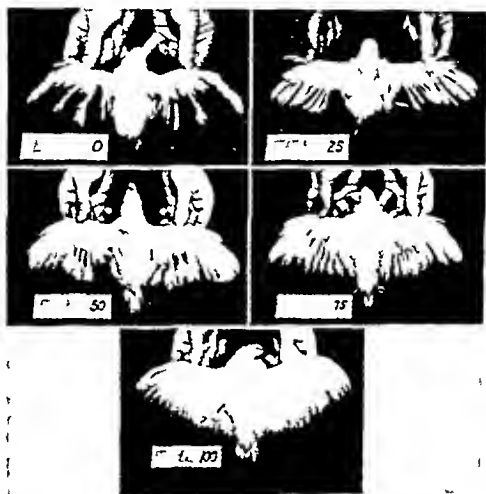


FIG 1 Feather scale 0 = very poor, 25 = poor, 50 = fair, 75 = good, 100 = very good

liver as the standard or by the improved method of Luckey *et al* (3) with both *Streptococcus lactis* R and *Lactobacillus casei* Bacterial activity is expressed in terms of micrograms of "folic acid" by giving the standard, solubilized liver, a potency of 1 and "folic acid" an assumed potency of 40,000 (2) Thus, solubilized liver is given a value of 25 γ of "folic acid" per gm We realize that this is purely an empirical method, because we do not know the exact amount of "folic acid" activity in solubilized liver and because the recent work of Stokstad (6) shows that crystalline liver "folic acid" has a relative potency of approximately 80,000 when compared to liver Fraction B When the amount of "folic acid" activity in solubilized liver is known, the figures which we have used may be corrected to the true amount by use of a simple proportion

Procedure for Making Liver Preparations—The Super Filtrol eluate is prepared according to the method of Hutchings, Bohonos, and Peterson (7). In brief, it is made by treating a solution (pH 3) of solubilized liver with norit and eluting the norit with a mixture of water, ethanol, and ammonia. Adsorption and elution are repeated with Super Filtrol in place of the norit and the eluate is concentrated under a vacuum. This preparation contains vitamin B₁₀, B₁₁, and "folic acid" activity and is used as the starting material for the majority of the preparations discussed in this paper. Approximately 20 gm of dry matter are obtained from 1 kilo of the starting material by this procedure and the active factors present are concentrated about 20-fold (owing to about a 60 per cent loss).

Preparation 52A (Containing Vitamins B₁₀, B₁₁, and "Folic Acid" Activity)—This fraction is made by adding 1500 cc of absolute ethanol to 500 cc of the Super Filtrol eluate equivalent to 1 kilo of solubilized liver and after this has stood in the cold the filtrate is acidified to pH 3 with sulfuric acid according to the technique of filtering, etc., outlined previously (1). The filtrate (Preparation 52A) is concentrated under a vacuum to the desired volume and neutralized. Further inactive material may be removed by adding acetone to the acid filtrate before concentration until a flocculent precipitate is formed. The filtrate is collected and concentrated in the same manner. Although we have used this same concentrate extensively, the procedure produces somewhat variable results.

*Alcohol-Soluble Butyl Ester*²—The starting material (generally Preparation 52A), equivalent to 1 kilo of solubilized liver, is dried carefully and placed in 5 liters of normal butyl alcohol (which then contains approximately 2 mg of dry matter per cc) and the solution is kept under nitrogen at 60° with constant stirring for 4 to 5 hours. The entire mixture is concentrated to dryness under a vacuum, extracted with 5 liters of absolute ethanol for 3 hours, and filtered. The alcohol-soluble portion is evaporated to dryness and extracted with 2 liters of water. The water-insoluble portion, containing the activity, is hydrolyzed under pressure, with 5 liters of a 1.5 per cent solution of ammonium hydroxide in a boiling water bath. The hydrolyzed material, now water-soluble, represents a concentration of vitamin B₁₁ approximately 140-fold from solubilized liver, and slightly less for vitamin B₁₀, however, an appreciable amount of "folic acid" activity was lost. When this preparation was fed to two different groups of chicks receiving the basal ration at a level equivalent to 8 per cent of solubilized liver (supplying 14 mg of dry matter per 100 gm of ration), normal growth and good feathers were produced (see Group 11, Table I).

Preparations 133C and 133D (Containing Principally Vitamin B₁₀)—

² This procedure may be found in detail in E. B. McQuarrie's Master's thesis, University of Wisconsin, 1943.

Preparation 133C is a 75 per cent ethanol precipitate (pH 7) of the 85 per cent ethanol precipitate (Preparation 2 of our previous report) of a modified Super Filtrol eluate. Preparation 133D is the precipitate obtained by acidifying to pH 3 the filtrate left from making Preparation 133C. Both of these precipitates are dissolved in water and made slightly basic with ammonium hydroxide and stored under toluene in the cold (as are all other preparations described in this paper). These fractions contain a large amount of vitamin B₁₀ activity in comparison to their content of vitamin B₁₁ and "folic acid."

Dialysis Procedure (Separation of Vitamins B₁₀ and B₁₁ from "Folic Acid" Activity)—Dialysis may be performed by placing the solution to be dialyzed in a closed cellophane bag in a large Soxhlet extractor with water. This permits the dialysate to be collected and concentrated and at the same time allows the bag to come in contact repeatedly with a new supply of hot distilled water. Treatment of the material to be dialyzed with taka diastase (20 mg of taka-diastase for each gm of dry matter and incubation at 37° for 24 hours under toluene) gives better separation. After dialysis "folic acid" activity occurs principally in the dialysate, while the greater part of vitamin B₁₀ and vitamin B₁₁ activity remains in the dialysis residue (Growth results with such fractions are presented later in the paper). This simple procedure concentrates vitamin B₁₀ and B₁₁ activity 4 fold from the Super Filtrol eluate. Dialysis may be carried out with similar results in cold running water if the dialysate is not desired.

Preparation 159R (Vitamin B₁₀ and B₁₁ Concentrate)—This preparation is the dialysis residue (see the dialysis procedure) of a calcium hydroxide filtrate of the Super Filtrol eluate. It contains nearly all of the vitamin B₁₀ and B₁₁ activity contained in the eluate but less than half of the "folic acid" activity.

Results

The results are given in Table I and are arranged in ascending order of vitamin B₁₀ activity to facilitate study of the table. Each entry is an average of results obtained with six chicks, except for the basal and control groups, which are an average of many groups. For the sake of brevity and simplicity, only about one-fourth of the results which we have obtained since our previous paper is presented. Groups of chicks whose response did not show a separation of any of the factors are omitted from Table I but the chemical properties of the factors as learned from such groups are presented later.

Separation of Vitamin B₁₀ Activity from Vitamin B₁₁ Activity—By comparing Column 4 with Column 5 (Table I) it is evident that, in confirmation of our previous results, the Super Filtrol eluate and certain liver fractions

contain at least two vitamins, vitamin B₁₀ for proper feather formation and vitamin B₁₁ for growth (note Groups 3 and 19, 9 and 10, 11 and 13, etc.) Since maximum feathering was not obtained without at least some growth

TABLE I

Results of Feeding Various Supplements to Basal Ration 486K (Given in Ascending Order of Feather Formation)

Group No	Preparation No and level fed equivalent to solubilized liver	Description of supplement (made from Preparation 52A* unless otherwise indicated)	Vitamin B ₁₀ activity (feather formation)†	Vitamin B ₁₁ activity (growth)	"Folic acid" activity per 100 gm ration	
					<i>Streptococcus lactis</i> R (6)	<i>Lactobacillus casei</i> (7)
(1)	(2)	(3)	(4)	(5)	(6)	(7)
				<i>per cent gain over basal†</i>	γ	γ
1	Basal ration	No supplement	30	0	0	0
2	128 \cong 10%	Methanol extract of Preparation 58 (cf Briggs <i>et al</i> (1))	35	-21	17	7
3	120R \cong 10%	Hot ethanol (100%) residue	40	70	7.5	15
4	101 \cong 5%	Methanol extract	45	40	25	
5	120E \cong 10%	Hot ethanol (100%) extract (see Group 3)	47	41	25	18
6	143F \cong 5%	Norit filtrate (pH 3) of Super Filtrol eluate*	50	55	9	16
7	113 \cong 5%	Norit (pH 3) eluate	60	-19	15	
8	102 \cong 5%	Methanol residue (see Group 4)	60	40	5	
9	133D \cong 5%	See text	70	2	5	1.6
10	111 \cong 5%	Super Filtrol (pH 3) eluate	70	96	16	
11	107 \cong 8%	Hydrolyzed butyl esters* (2 groups of chicks)	75	110	8.3	3.3
12	139E \cong 5%	Norit (pH 10) eluate of Super Filtrol eluate	75	55	17.5	20
13	133C \cong 5%	See text	75	32	0.6	0.4
14	78R \cong 5%	Made same as Preparation 59 (cf (1))	75	101	5	
15	161P \cong 10%	Lead ppt of Preparation 159R*	93	65	6	6
16	30-2 \cong 8%	Made same as Preparation 30 (cf (1))	95	115	8	3
17	162P \cong 10%	Zinc ppt of Preparation 159R*	95	69	5	4
18	138R \cong 5%	Hot ethanol (95%) residue of Super Filtrol eluate	95	87	40	15

TABLE I—Concluded

Group No	Preparation No and level fed equivalent to solubilized liver	Description of supplement (made from Preparation 52A* unless otherwise indicated)	Vitamin B ₁₀ activity (feather formation)†	Vitamin B ₁₁ activity (growth)	"Folic acid" activity per 100 gm ration	
					<i>Streptococcus lactis</i> R (6)	<i>Lactobacillus casei</i> (7)
(1)	(2)	(3)	(4)	(5)	(6)	(7)
				per cent gain over basal‡	γ	γ
19	79 ≈5%	1st norit eluate of solubilized liver	100	42	42	
20	150 ≈10%	Butanol residue	100	102	25	15
21	146 ≈10%	75% ethanol (pH 3) residue of Super Filtrol eluate	100	128	15	36
22	52A ≈5%	See text	100	102	24	25
23	Control ration, 2% solubilized liver		100	100	50	50

* See the text for information regarding the starting material

† 0 = very poor, 25 = poor, 50 = fair, 75 = good, 100 = very good

‡ $\frac{\text{Gm gain over basal} \times 100}{(\text{Control weight}) - (\text{basal weight})}$

The average control weights at 4 weeks range from 200 to 275 gm and the average basal weights range from 100 to 150 gm, depending on the batch of chickens

increase, we cannot definitely conclude that vitamin B₁₀ is not utilized in part for growth as well as for feathering. Likewise, since maximum growth was not obtained without at least "good" feathers, it is impossible to decide at the present time whether or not vitamin B₁₁ also improves feather formation to some extent (even in the absence of vitamin B₁₀). Thus, although the principal effects of the two vitamins are entirely different, it is conceivable that there may be some overlapping in their action (especially if bacterial synthesis within the intestine is considered).

Separation of Vitamin B₁₀ and B₁₁ Activity from "Folic Acid" Activity—In confirmation of our earlier results, the two vitamins are distinct from "folic acid" activity (compare Columns 4 and 5 with Columns 6 and 7). Maximum or near maximum feather formation could be obtained (Groups 13 to 17) with lower amounts of "folic acid" activity than those which gave little feather formation (Groups 2 to 6). Likewise normal or near normal growth could be obtained (Groups 11, 14, and 16) with lower amounts of "folic acid" than those which gave only small increases in growth (Groups 2, 4, 5, 7, 12, and 19). We recognize the fact that there is a general tendency for supplements which have high vitamin B₁₀ and B₁₁ activity to have high "folic acid" activity, however, such supplements are usually those that have undergone little purification.

Separation of Streptococcus lactis R from Lactobacillus casei Activity—

When Column 6 is compared with Column 7, it is evident that "folic acid" activity as measured by the two organisms does not correlate (Compare Groups 2 and 3, 6 and 16, 20 and 21, etc.) Nor does the activity of either one correlate any better than the other with growth or feathering. Thus, there are at least four biologically active compounds in the Super Filtrol eluate of liver, the significance of which will be discussed later in the paper.

In regard to the requirement of the chick for "folic acid," we have stated in our previous paper (1) that "maximum growth was not obtained in chicks unless levels were fed equal to, or above, 17.5 γ of folic acid per 100 gm of ration." In Table I it is seen that maximum growth may be obtained with smaller amounts of "folic acid" than this, namely, 5 γ per 100 gm of ration as measured by *Streptococcus lactis* R (Group 14) or 3 γ per 100 gm of ration as measured by *Lactobacillus casei* (Group 16). Near maximum feather formation may be obtained with similar amounts of "folic acid" activity (see Groups 16 and 17). On the other hand various supplements which supplied greater amounts of "folic acid" were low or devoid of growth and feathering activity (Groups 2 to 5 and Group 7). Thus, we may conclude, that liver "folic acid" as we measure it is not needed *per se* by the chick for growth or feathering unless in very small amounts.

Further Description of Deficiency Symptoms and Other Results—When chicks are about 1 week of age, the innermost secondary wing feathers begin to curl toward the body and the wing coverts (the feathers covering the quills) begin to curl outward, giving the chick a ruffled appearance. The shafts of many wing feathers appear narrower than normal shafts and the feathers are frequently broken off. By the time the chick is 4 weeks old, the wing feathers remaining are either stunted or have scanty barb formation. They are curled outward, and frequently have a malformed shaft (often completely twisted around). Body and tail feathers are slow in appearing and are of abnormal structure (see Fig. 1).

An anemia (macrocytic), as mentioned previously (8), occurs in chicks on the basal ration. Any supplement which improves growth or feathering when fed with the basal ration likewise appears to correct the anemia partially, so it is possible that both vitamins are necessary for proper blood formation. As will be seen in Table II, the anemia-preventing activity can be separated from "folic acid" activity as measured with both *Streptococcus lactis* R and *Lactobacillus casei*. Preliminary studies showed that deficient chicks also have leucopenia. The paralysis and the perosis, as observed previously (1) in chicks receiving the basal ration, still occur occasionally.

Among the various compounds which have been tested for vitamin B₁₀ and B₁₁ activity and found to be inactive are *dl*-lysine, *l*-tryptophane, *d*-glutamic acid, *l*-aspartic acid, asparagine, glutamine, pimelic acid, yeast

nucleic acid, xanthine, guanine, uracil, orotic acid, adenine, adenylic acid, xanthopterin, adenyliothiomethylpentose, and *m*-aminobenzoic acid. The level of known vitamins was doubled in the ration with no effect. Likewise, the addition of small amounts of inorganic salts of cobalt, boron, silicon, nickel, molybdenum, and aluminum was ineffective, as was the ash of solubilized liver.

The following substances have varying growth-promoting or feather-forming activity, but because they occur in only trace amounts in the eluate they cannot be either vitamin B₁₀ or B₁₁. *p*-aminobenzoic acid (5 to 10 mg per 100 gm of ration) (5), ascorbic acid (100 mg per 100 gm of ration) (9), thymine (200 γ per 100 gm of ration), and thymus nucleic acid (20 mg per 100 gm of ration). We do not routinely add any of these compounds to our ration because their action may be indirect and would con

TABLE II

Results of Dialysis Procedure on Separation of Factors Necessary for Feather Formation, Growth, "Folic Acid" Activity, and Anthanemia Activity

Supplement to basal Ration 486K	No dead at 4 wks (24 chicks at start) per group	Vitamin B ₁₀ activity (feather formation)*	Vitamin B ₁₁ activity (growth)*	Folic acid 'activity per 100 gm ration		Hemoglobin (12 chicks)
				<i>Streptococcus lactis</i> R	<i>Lactobacillus casei</i>	
Dialysate (average of 4 preparations)	1	38	39	18.4	11.5	7.26
Dialysis residue (average of 4 preparations)	0	82	79	13.1	10.1	9.11

* See Table I

fuse the results. The effects obtained by feeding sulfasuxidine to chicks receiving this ration have been presented (5).

Properties of Vitamin B₁₀ and Vitamin B₁₁—The properties of these two vitamins are considered together, since they are similar and separated only with difficulty. Unless otherwise indicated, all tests were made on fractions obtained from solubilized liver which were concentrated 20 to 100 times. (The Super Filtrol eluate was used most generally.) All fractions were tested for "folic acid" activity but, since descriptions of many properties of "folic acid" have been published (4, 7, 10), only those results will be given which have a direct bearing on the immediate problem. It is important to bear in mind that properties of the vitamins may vary, depending on the concentration of the solution used.

Solubility—In general, both vitamins B₁₀ and B₁₁ are insoluble in common organic solvents such as ether (neutral or pH 3), butyl alcohol (neutral,

pH 10, or pH 3), acetone, ethyl acetate, and 95 per cent and absolute (cold) ethanol. If hot absolute ethanol is used as the solvent, "folic acid" activity for *Streptococcus lachis* R is largely removed, leaving vitamin B₁₁ mainly in the residue, while vitamin B₁₀ and "folic acid" activity for *Lactobacillus casei* are in both extract and residue. The two vitamins are less soluble in 90 per cent ethanol (pH 3) and 85 per cent ethanol than is "folic acid" activity. Also, at such concentrations (75 to 85 per cent) vitamin B₁₀ tends to be more insoluble in ethanol than vitamin B₁₁. Both vitamins are soluble in water and glacial acetic acid. Vitamin B₁₁ is slightly more soluble in methanol than vitamin B₁₀, however, both vitamins are less soluble than "folic acid" activity.

Stability—Vitamins B₁₀ and B₁₁ as they occur in the eluate are completely destroyed by autoclaving at 15 pounds pressure in 2 N hydrochloric acid for 1 hour, although they remain stable when autoclaved at pH 3 for the same time. Autoclaving with 1 N sodium hydroxide for 30 minutes destroys little vitamin B₁₀ and B₁₁ activity, while preparations autoclaved at pH 10 or 7 for 30 minutes retain all measurable amounts of activity. Both vitamins and "folic acid" are stable to dry heat (110°) for 24 hours.

Oxidation with a 2 per cent solution of hydrogen peroxide for 2 hours (hot) destroys most of the vitamin B₁₀ and "folic acid" activity but about 50 per cent of the vitamin B₁₁ activity remains. Bubbling hydrogen through a solution of the vitamins for 10 hours causes no destruction of any of the three factors. Nitrous acid treatment (5 per cent solution of nitrous acid at room temperature for 12 hours) causes destruction of nearly all biological activity, indicating the presence of active amino groups in all of these compounds.

Other Properties—Both vitamins are adsorbed to the greatest extent in acid solution (pH 3 is used) on fullers' earth, norit, and Super Filtrol. Vitamin B₁₀ is adsorbed on norit more completely than vitamin B₁₁ and it is also eluted more rapidly (with the alcohol, ammonia, and water mixture), which suggests the possibility of separating these two vitamins from each other by chromatographic adsorption.

Both vitamins and "folic acid" activity are precipitated completely by lead acetate and zinc chloride and partially with silver nitrate and barium hydroxide (the water-soluble barium salts are insoluble in 80 per cent ethanol). The activity is not precipitated by calcium hydroxide.

The butyl esters of both vitamins can be made by the procedure described in this paper, which indicates that both of these vitamins have acid groups. That acid groups are present and may even predominate is also suggested by the fact that both vitamin B₁₀ and vitamin B₁₁ migrate toward the anode during electrophoresis. The activity of all three factors was concentrated in an acid cell (pH 2.1) of a five cell electro dialysis apparatus.

Dialysis procedures have produced very interesting results, which are given in Table II. It can be seen that both vitamins B_{10} and B_{11} (and antianemic activity) do not pass readily through cellophane membranes. On the other hand "folic acid" activity for both bacteria occurs principally in the dialysate. Results are especially clear cut when the fraction has been previously treated with taka-diastase (see the dialysis procedure).

DISCUSSION

The importance of vitamin B_{10} and vitamin B_{11} in the nutrition of other animals besides the chick has not been determined as yet. However, the Super Filtrol eluate, containing both of these vitamins (as well as "folic acid" activity) has been shown to be important for the nutrition of the rat (11-13), dog,³ and monkey (14). The growth activity of this fraction has been largely attributed to its "folic acid" content but our results with the chick suggest that vitamins B_{10} and B_{11} may be as important or more so.

The relationship of these two vitamins to other unknown chick factors has been discussed in our previous paper (1). However, work done since that time changes the picture slightly. Pfiffner *et al* (15) reported the isolation from liver of an antianemia factor for chicks, called vitamin B_c , in yellow crystalline form. The method of isolation and rations used are unpublished at the present time. The vitamin prevented anemia in chicks and caused the chicks to "grow normally" when fed at a level of 250 γ (probably an excess) per 100 gm of ration. The authors state that they "demonstrate conclusively the identity of Hogan's antianemia factor and Peterson's 'eluate factor'" (16) and state that these factors are probably the same as "folic acid". If this is true, the results presented in this paper indicate that the crystalline vitamin B_c of Pfiffner *et al* is distinct from true vitamins B_{10} and B_{11} as they occur in the Super Filtrol eluate. Furthermore, it is also important to note (Table II) that the antianemia activity in our work remains in the undialyzable portion and does not follow liver "folic acid" activity. Later, a report by O'Dell and Hogan (17) presented some chemical properties of vitamin B_c (in impure concentrates) and gave a technique for its assay. They do not give, however, the effects of their factor upon growth or feathering when fed in addition to their improved purified diets. From the properties ascribed to vitamin B_c by O'Dell and Hogan (such as solubility in methanol, incomplete adsorption on fullers' earth at acid pH, and stability to oxidation, etc.) it would appear that their vitamin is more similar to vitamin B_{11} than to vitamin B_{10} .

A review of the literature on "folic acid" has been made by Luckey *et al* (3) but it is important to mention the report of Stokstad (6) who isolated

³ Krehl, W. A., and Elvehjem, C. A., unpublished.

a compound from liver (thought to be identical with the crystalline compound of Pffner *et al* (15)), and also isolated a different compound from yeast. This latter compound had about one-half the activity for *Streptococcus lactis* R as the liver compound but about the same amount of *Lactobacillus casei* activity. We have not as yet tried either of these isolated substances for vitamin B₁₀ or vitamin B₁₁ activity. However, from the results which we have presented in this paper we would expect the liver compound to be largely inactive. In this connection Almquist has recently reported (18) that a purified fraction of the "folic acid" for *Lactobacillus casei* obtained from Stokstad had little, if any, activity for the chick and states, in agreement with our previous results (1), that "at least one unknown member of the B-complex is required by chicks" other than the *Lactobacillus casei* factor. It is interesting that Daft and Sebrell have reported (19) that the several "folic acids" (crystalline) had activity in the prevention of granulocytopenia and leucopenia in rats fed sulfonamides.

The results which we have obtained with the dialysis procedures may indicate that the various "folic acids" as measured by *Streptococcus lactis* R and *Lactobacillus casei* are fragments of a large molecule, or molecules, which are needed by the chick in the intact form for true vitamin B₁₀, vitamin B₁₁, and antianemia activity. Thus, the possibility exists that vitamins B₁₀ and B₁₁ may have some "folic acid" activity. Similarly it is entirely possible that pure compounds showing high activity for either *Streptococcus lactis* R or *Lactobacillus casei* may have some vitamin B₁₀ or vitamin B₁₁ activity but the action may be indirect. Work to answer this question and to separate and concentrate vitamins B₁₀ and B₁₁ further is in progress at the present time.

SUMMARY

Various chemical properties for vitamins B₁₀ and B₁₁ as well as methods for their partial separation are described.

At least four substances with biological activity, namely vitamin B₁₀ (necessary for feather formation in chicks), vitamin B₁₁ (necessary for growth), and two factors necessary for *Streptococcus lactis* R and *Lactobacillus casei*, are present in the Super Filtrate eluate of solubilized liver. The significance of this is discussed.

"Folic acid" activity has been separated, at least in part, from vitamin B₁₀, B₁₁, and antianemia activity and does not appear to be necessary *per se* for the chick unless in small amounts.

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THE LIPID, SODIUM, CHLORIDE, AND NITROGEN CONTENT OF THE RESPIRATORY TRACT FLUID OF NORMAL ANIMALS

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Expectorants are drugs which are reputed to affect the volume or composition or both of secretions produced in the respiratory tract and to which the term respiratory tract fluid has been assigned in this laboratory. The dearth of experimental evidence upon the pharmacology of expectorants being recognized, studies were begun 5 or 6 years ago and, after some time, a method was finally developed for collecting the fluid (1). In the present communication will be described analyses of the respiratory tract fluid of normal, urethanized dogs, cats, rabbits, and cockerels for lipids, sodium, chloride, and nitrogen, with some observations upon the specific gravity and viscosity of this fluid.

Respiratory tract fluid was collected through a tracheal cannula ligated into a healthy, adult, urethanized animal, and with the inhaled air conditioned to body temperature and approximately 100 per cent relative humidity according to the method now in use in this laboratory (2). Lipids were estimated by the oxidative micro technique (3), sodium by the method of Hoffman and Osgood (4), chloride by Van Slyke's method (5), and protein and non-protein nitrogen by the method of Conway (6).

The *lipid composition* of the fluid of twenty-seven normal, urethanized rabbits, cats and dogs has been summarized and the data presented in Table I. Respiratory tract fluid was found to contain all of the lipids found in blood by the same technique, though in much smaller amounts. The total lipid of the fluid in cats and rabbits averaged about the same, some 60 mg per 100 ml of fluid, while the fluid of the dog contained about 3 times this amount of total lipid. The percentage composition of the total lipid was similar to that found in blood plasma by the same technique.

The *sodium and chloride content* of the fluid of over 100 rabbits, cats, and dogs has been summarized in Table I. The mean sodium values were fairly constant among the three species at about 30 to 40 mg per 100 ml of respiratory tract fluid, though individually the values varied considerably, as is apparent in the relatively high figure for the standard error of each mean. Also the mean chloride content was of the same order in rabbits, cats, and dogs, at some 50 to 70 mg per 100 ml. These values for sodium

and chloride are considerably smaller than those of blood plasma, though there is roughly the same relative relation of one to the other. In other words, sodium and chloride are present in the fluid roughly in the proportion of mole for mole.

The above considerations suggest that, in so far as lipids, sodium, and chloride are concerned, respiratory tract fluid might be regarded as a diluted filtrate of blood plasma. The impression is not intended to be conveyed that the fluid as a whole may be considered a diluted filtrate of blood plasma, but merely that such may be the case with respect to lipids, sodium, and chloride. To investigate this further, the mean content of these substances in blood plasma was compared with corresponding means for the fluid. The mean plasma lipid content was taken from figures previously published by Boyd (7) with respect to rabbits and cats, and from unpublished reports in connection with dogs,¹ all values being obtained by the same analyst using the same oxidative micro technique. The mean value of plasma sodium was obtained by the same analyst (M. S.) using the same technique upon fifteen normal rabbits, twelve cats, and twelve dogs. The mean value for plasma chloride was also obtained by the same analyst (M. M.) using the same technique upon twelve normal rabbits, five cats, and eleven dogs.

The mean values for the various constituents of the fluid were then expressed as a percentage of the mean values for the corresponding constituents of blood plasma. It was found that the constituents of the fluid bore no constant relationship to the constituents of blood plasma. Thus, for example, the lipid content of the fluid of the rabbit varied from 17 to 36 per cent of the lipid content of rabbit plasma. It may be concluded that while simple diffusion may play a part in the formation of the fluid selective secretion is also active. The well known presence of glands along the respiratory tract would also suggest that the fluid is a secretion. Frankly, however, the relative part played by acinar glands, goblet cells, other mucosal cells, and the epithelium of the alveoli and related structures in the production of respiratory tract fluid has not yet been proved.

The *protein and non-protein nitrogen* content of the fluid of nineteen rabbits, cats, and cockerels has been summarized in Table I. The mean values were found to be considerably lower than in plasma.

In most of the above work, the chemical analyses were performed upon the total sample of fluid which had collected by the end of the day or over night. It was possible that the composition might change as the experiment continued from hour to hour, however. Since very little material is needed for the nitrogen determinations by Conway's method, it was decided to follow the nitrogen levels from hour to hour and compare them with the volume output of the fluid in ml. per kilo per 24 hours. These experiments

¹ Boyd, E. M., unpublished data.

were performed upon ten rabbits and the mean hourly data are collected in Table II. It may be seen that there was some increase in the concentration

TABLE I

Composition of Respiratory Tract Fluid of Normal Animals

The results, except for specific gravity and relative viscosity, are expressed as the mean \pm the standard error in mg per 100 ml of respiratory tract fluid

Constituent	No of animals				Rabbit	Cat	Dog	Cockerel
	Rabbit	Cat	Dog	Cockerel				
Total lipid	13	9	5		65 \pm 6.7	60 \pm 1.4	197 \pm 22	
Neutral fat	13	9	5		18 \pm 6.9	12 \pm 2.2	53 \pm 14	
Total fatty acids	13	9	5		42 \pm 4.7	33 \pm 2.7	126 \pm 18	
“ cholesterol	13	9	5		13 \pm 6.1	19 \pm 2.5	41 \pm 7.4	
Ester “	13	9	5		8 \pm 3.9	12 \pm 0.7	28 \pm 9.2	
Free “	13	9	5		5 \pm 1.1	7 \pm 1.8	13 \pm 1.8	
Phospholipid	13	9	5		28 \pm 1.0	22 \pm 3.3	85 \pm 17	
Sodium	13	18	10		36 \pm 7.8	42 \pm 9.5	33 \pm 7.3	
Chloride	29	58	5		46 \pm 5.4	66 \pm 4.7	66 \pm 16	
Non protein N	10	5	4		6.3 \pm 0.8	3.5 \pm 1.6		3.0 \pm 1.5
Protein N	10	5	4		10.4 \pm 2.1	13.6 \pm 3.7		26 \pm 4.0
Sp gr	13	56			0.985 \pm 0.006	1.014 \pm 0.006		
Relative viscosity	12	25			1.02 \pm 0.01	0.95 \pm 0.02		

TABLE II

Mean Hourly Changes in Volume, Protein, and Non Protein Nitrogen Content of Rabbit Respiratory Tract Fluid

Hr	Volume	Protein N	Non protein N
	<i>ml per kg per 24 hrs</i>	<i>mg per 100 ml</i>	<i>mg per 100 ml</i>
1	2 0	7 62	4 13
2	2 4	7 75	3 68
3	2 6	8 98	5 46
4	2 6	5 50	7 49
5	1 9	11 8	8 71
6	2 6	13 3	6 76
7	2 4	13 7	5 57
8	1 7	12 7	8 10

of protein nitrogen as the experiment progressed but little change in the concentration of non-protein nitrogen. These experiments emphasize the desirability of having controls arranged side by side with each animal in

experiments on the effect of any factor upon the composition of the fluid, rather than having the initial fluid of an animal serve as its own control

The *specific gravity and relative viscosity* of the fluid of normal, urethanized cats and rabbits has been summarized in Table I. Relative viscosities were determined in the Ostwald viscosity pipette. It is obvious from the data given in Table I, that the specific gravity and relative viscosity of respiratory tract fluid are near that of distilled water in both rabbits and cats

SUMMARY

Values are given for the lipid composition of the respiratory tract fluid of 27 normal, urethanized rabbits, cats, and dogs, the chloride content of 29 rabbits, 58 cats, and 5 dogs, the sodium content of 13 rabbits, 18 cats, and 10 dogs, the protein and non-protein nitrogen content of 10 rabbits, 5 cats, and 4 cockerels, the specific gravity of 56 cats and 13 rabbits, and the relative viscosity of 25 cats and 12 rabbits

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A PHOTOMETRIC METHOD FOR THE DETERMINATION OF CHOLATES IN BILE AND BLOOD*

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Reinhold and Wilson (1) have described a modification of the method of Gregory and Pascoe (2) which permits the determination of cholic acid in pure solutions and in bile with an accuracy approaching the maximum that can be attained with the visual color comparator. By this procedure cholates (glycocholate, taurocholate, and unconjugated cholate) can be determined with specificity even in the presence of dihydroxy- and mono-hydroxycholanates. Modifications of this method have been described (3-6), but a critical evaluation of essential factors in the method is desirable. Procedures for the determination of cholate involving other color reactions (7-9) do not appear to offer advantages in specificity or in the accuracy of analytical recoveries.

There is much uncertainty regarding the question of the presence of bile salts in the peripheral circulation of normal animals (10). Employing modifications of the method of Gregory and Pascoe, Josephson (3) reported that the normal concentration of cholate in blood is 0.5 to 2 mg per 100 ml, and Schmidt (5) stated that the concentration is less than 2 mg per 100 ml. By the use of another method, Perlzweig and Barron (11) were unable to demonstrate the presence of bile salts in normal blood. This was in agreement with the report of Herzfeld and Haemmerle (12). Greene and Aldrich (13) and Aldrich and Bledsoe (14), by the use of a modified Pettenkofer reaction, found normal concentrations of bile salts in blood ranging from 3 to 6 mg per 100 ml. With a method which was stated to be specific for the bile acid *group* of compounds but not for any *particular* bile acid, Tashiro (15) reported normal concentrations of 60 to 100 mg per 100 ml of blood. These analytical uncertainties are emphasized by reports of unsuccessful attempts to isolate bile acids from blood obtained from the peripheral circulation of normal animals (10).

In this paper the method of Reinhold and Wilson is adapted for the photoelectric filter photometer. Certain modifications in the conditions for the color reaction are employed in order to secure the maximum increase

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in accuracy attainable with the filter photometer. With such changes the method is applied with satisfactory accuracy to the determination of cholate in bile. Two procedures are described for the preparation of filtrates and extracts of blood for photometric estimation of the concentration of cholate. The accuracy of the method as applied to the determination of cholate in blood is limited by the low concentrations of this bile salt and by losses in the preparation of filtrates and extracts. However, we believe that the method represents a definite improvement in the specificity and accuracy of estimations of blood cholate. Preliminary experiments are described in which this method is applied to a study of the concentrations of cholate in the peripheral circulation of normal animals and of those with biliary obstructions.

EXPERIMENTAL

Preparation and Purification of Bile Acids for Use As Analytical Standards—Cholic acid was isolated from ox bile and was purified as described by Sobotka (16). Glycocholic and taurocholic acids were synthesized from cholic acid by the procedure of Cortese *et al* (17). Other samples of these conjugated bile acids were isolated from bile and purified as described by Hammarsten (18). The purity of all samples was verified by determinations of melting points, solubilities, and optical rotations and by elementary analyses.

The primary standard for all analyses and experiments was a highly purified sample of crystalline cholic acid. Inasmuch as cholic acid was used as the primary standard for the analysis of solutions containing both glycocholic and taurocholic acids (or their salts) as well as solutions of cholic acid (or cholate salts), it seemed most satisfactory to express cholate concentration in terms of millimolarity. This was adopted for recording the results of analyses of bile. However, the concentrations of cholates in blood are of such small magnitude that it was more convenient to express them as *mg of cholic acid per 100 ml of blood*. The use of the latter unit involves no implication regarding the form of cholate present, whether "free" or conjugated with glycine or taurine.

Procedure for Color Development and Preparation of Extinction-Concentration Curve—In succeeding sections of this paper there are outlined the results of experiments which provide information concerning optimum conditions for color development with cholic acid in the furfural-sulfuric acid reaction. The procedure finally adopted is illustrated in the following description of the determination of the relationship between extinction and the concentration of cholate. In all analyses reported in this paper the conditions for color development were identical with those described in the following section except where experimental changes are specifically designated.

Reagents—

1 16.0 N sulfuric acid This reagent is prepared from c. p. sulfuric acid, and the concentration is adjusted accurately

2 1 per cent furfural solution 10 ml (20°) of pure furfural (freshly redistilled) is dissolved in distilled water, and the volume is brought to 100 ml

3 Glacial acetic acid, c. p.

In test-tubes of uniform size (18 mm inside diameter) are placed 1 ml portions of solutions of sodium cholate (prepared from the standard sample of pure cholic acid) with a range of concentrations equivalent to 0.02 to 0.3 mg of cholic acid per ml. To each of these samples are added 6 ml of 16 N sulfuric acid and 1 ml of 1 per cent furfural solution. At the same time reagent blanks are prepared in separate test-tubes by substituting 1 ml of distilled water for the cholate solution in mixtures containing the other reagents in their usual proportions. Standard and blank tubes are heated simultaneously for exactly 13 minutes in a water bath maintained at 65° (controlled to $\pm 0.1^\circ$). At the end of the period of heating, the tubes are cooled rapidly to room temperature by immersion in a cold bath. To the cooled solutions are added 5 ml portions of glacial acetic acid. The transmittancies of the standard solutions are determined in a photoelectric filter photometer (19) at 620 and 660 $m\mu$ ¹ with the transmittance of the blank set at unity. In Fig. 1 the extinctions² are plotted against the corre-

¹ These are the mean wave lengths of the transmission bands of the filters. The transmission limits of these filters are from 595 to 660 $m\mu$ and from 635 to 720 $m\mu$, respectively. Throughout this paper, wave length designations refer to the mean wave lengths of the transmission bands of the particular filters employed.

² Extinction, ϵ' , is defined as $-\log T$, where T is the transmittance expressed as a fraction of unity. The concentration, C_u , of cholate in an unknown solution is obtained by the equation, $C_u = \epsilon'_u/\epsilon_s$, where ϵ_u is the extinction of the colored solution obtained with the unknown quantity of cholate and ϵ_s is the calculated extinction for a colored solution prepared from standard cholate of unit concentration (1 mg of cholic acid per ml). ϵ_s is calculated from extinction-concentration data (Fig. 1). The calculated values of ϵ_s as determined with our photometer (Evelyn type (19)) were 2.62 ± 0.02 for 620 $m\mu$ and 2.89 ± 0.03 for 660 $m\mu$. Values of ϵ_s as determined with a filter photometer are dependent upon the transmission characteristics of the filters and upon the effective depth of solution through which the "light" passes. This depth is difficult to designate precisely for photometers of the Evelyn type in which the slits are wide and in which cylindrical test tubes are used as cuvettes. For these reasons, calibration data (extinction-concentration relationships) should be determined for each instrument.

An alternative method may be employed for the determination and calculation of cholate concentrations. According to this procedure, a standard solution of cholate is carried through the reaction for color development along with each group of cholate solutions of unknown concentrations. From the respective transmittancies of the solutions, the corresponding extinctions for the standard, ϵ'_s , and the unknown, ϵ'_u , are calculated. The concentration of cholate in the unknown solution, C_u , is calcu-

sponding concentrations of cholate in the original standard aqueous solutions (expressed as mg of cholic acid per ml) Beer's law is applicable over a satisfactory range of concentrations. Because of the marked effect of the concentration of sulfuric acid used in the color reaction, calibration data should be obtained with each new stock of reagent unless great care is taken to adjust the concentration to exactly 16 N each time. In Table I data are recorded for analyses of pure solutions of cholates by this method.

Selection of Optimum Conditions—Before adoption of the procedure for color development described in the preceding section, a number of experi-

TABLE I

Analyses of Solutions of Pure Cholate, Glycocholate, and Taurocholate Salts

The analytical figures are averages of five determinations. The average error of a single determination, a , is calculated from the equation, $a = \pm \Sigma v/n$, where Σv is the sum of all the deviations from the average, and n is the number of determinations.

Type of bile salt	Concentration (calculated)	Average concentration (determined)	Average analytical recovery	Average error of single determination
	mm	mm	per cent of calculated	per cent
	2.40	2.38	99.2	1.4
	5.00	5.02	100.4	1.2
Glycocholate	1.00	0.99	99.0	1.6
	2.00	2.01	100.5	1.5
Taurocholate	1.00	0.97	97.0	1.9
	2.00	1.97	98.5	1.6
Mixture of				
Cholate	1.20	3.17	99.1	1.6
Glycocholate	1.00			
Taurocholate	1.00			

ments were performed for the selection of optimum conditions. These experiments are summarized by Figs. 2 to 6 and by the following statements.

(a) *Optimum Concentration of Sulfuric Acid*—The concentration of sulfuric acid in the reaction mixture has a marked effect on the specificity of the reaction, on the hue of the color (as determined by wave length transmittance curves), and on the relationship between extinction and the concentration of cholate. The optima for temperature and duration of the reaction also are influenced by the concentration of sulfuric acid. Study

lated from the concentration of the standard, C_s , by the equation $C_u = C_s \cdot A_u/A_s$. Since, by this method, standard and unknown solutions are treated under identical conditions, this procedure is useful when instruments for the precise control of time and temperature of reaction are not available.

of these several relationships for a variety of concentrations of this reagent led to the adoption of the concentration recommended by Reinhold and Wilson (1), *i.e.*, 12 N in the reaction mixture (based on the volume of solution before the terminal dilution with glacial acetic acid). This concentration is attained by the use of the 16 N sulfuric acid reagent in the proportions stated above.

(b) *Abridged Wave-Length-Transmittance Curve*—Fig. 2 is an abridged wave-length-transmittance curve for the pigment produced in the reaction described above. Data for this curve were obtained with an Evelyn filter

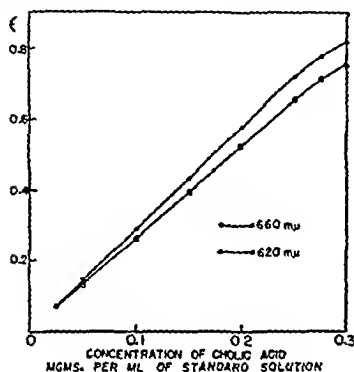


FIG. 1

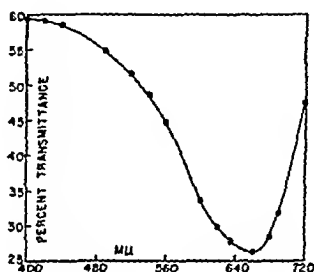


FIG. 2

FIG. 1 Extinction-concentration relationships for the furfural-sulfuric acid reaction for cholic acid. Concentrations refer to the cholic acid equivalents of the standard aqueous solutions of sodium cholate which are used for color development. Inasmuch as 1 ml. portions of these solutions are used for color development, the concentration figures are the total quantities (mg.) of cholic acid contained in the final colored solutions.

FIG. 2 Abridged wave length per cent transmittance curve for the pigment produced from cholic acid (0.2 mg.) in the modified furfural-sulfuric acid reaction.

photometer, and wave-length designations are the mean values of the transmission bands of the filters. Although minimum transmittance occurs at 660 mμ, a slight advantage in precision is attained by determining cholate concentrations from measurements at 620 mμ for a reason which is stated in a succeeding paragraph.

(c) *Optimum Temperature and Reaction Time* (See Figs. 3 and 4)—Although attainment of minimum transmittance at 620 and 660 mμ requires a longer reaction period at 65° than at 70°, greater precision can be obtained at the lower temperature, as suggested by the slower rate of increase in transmittance beyond the minimum point. For a similar reason, a slight advantage in precision is attained by calculation of cholate concentrations

from transmittance measurements at 620 $m\mu$ rather than 660 $m\mu$. Data for time and temperature may be applied directly only when 18 mm test-tubes are used, but the general relationships are independent of this factor.

(d) *Optimum Concentration of Furfural*—The data of Fig 5 demonstrate that minimum transmittancies at 620 $m\mu$ are obtained when the concentration of furfural in the reaction mixture is 0.12 to 0.15 per cent by volume (based on the volume of the reaction mixture before the terminal dilution with glacial acetic acid). A standard concentration of furfural of 0.125 per

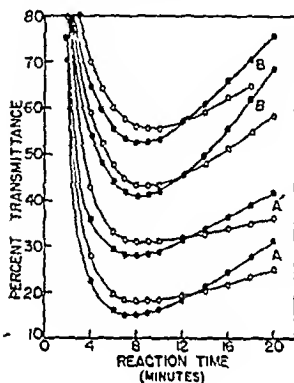


FIG 3

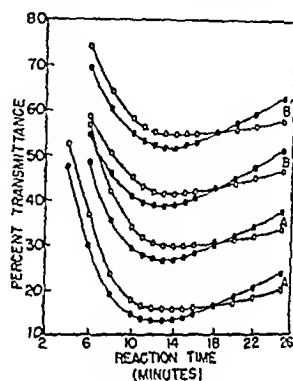


FIG 4

FIG 3 Per cent transmittance reaction time curves for the modified Gregory Pascoe reaction at 70°. The quantities of cholic acid used for color development were 0.2 mg for Curves A and A' and 0.1 mg for Curves B and B'. Data for Curves A and B were obtained on solutions from which glacial acetic acid was omitted, for Curves A' and B', on corresponding solutions which were diluted with 5 ml portions of glacial acetic acid. Curves designated by the symbol O are per cent transmittancies at 620 $m\mu$, and those designated by the symbol ● are measurements at 660 $m\mu$.

FIG 4 Per cent transmittance reaction time curves for the modified Gregory Pascoe reaction at 65°. The curves are labeled in a manner corresponding to those of Fig 3.

cent by volume was adopted for all reactions, and this is accomplished by use of the 1 per cent reagent as described above.

(e) *Changes in Transmittance after Color Development*—Fig 6 provides information concerning the stability of the pigment at room temperature (23°). Dilution of the solutions with glacial acetic acid after termination of the reaction minimizes change in transmittance. In addition, it diminishes or prevents turbidity which otherwise develops occasionally in analyses of filtrates or extracts of bile and blood.

(f) *Specificity of Color Reaction*—Reinhold and Wilson (1) investigated

the specificity of the reaction by trials upon a variety of compounds. We have verified their conclusions and have extended the investigation. It is particularly important to note that in the bile acid series, desoxycholic, chenodesoxycholic, α -hyodesoxycholic, β -hyodesoxycholic, lithocholic, dehydrocholic, and 3-hydroxy-6-ketoallocholic acids do not yield color in the modified reaction. Thus, in this group of compounds, the reaction appears to be specific for 3,7,12-trihydroxycholic acid, either unconju-

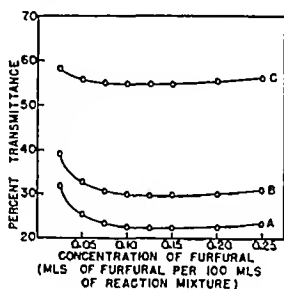


FIG 5

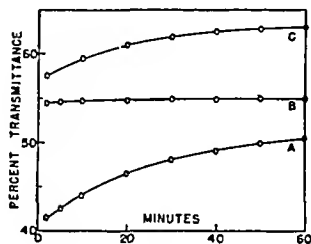


FIG 6

FIG 5 Effect of variations in the concentration of furfural in the reaction mixtures (temperature of reaction 65° , duration 13 minutes). The concentrations are expressed as ml of pure furfural per 100 ml of the reaction mixtures (based on the volumes before dilution with glacial acetic acid). However, glacial acetic acid was added to the final colored solutions before the transmittancies ($620 m\mu$) were determined. Reactions were conducted with the following quantities of cholic acid: Curve A, 0.25 mg; Curve B, 0.2 mg; Curve C, 0.1 mg.

FIG 6 Changes in per cent transmittancies ($620 m\mu$) of the colored solutions during periods of standing at room temperature after completion of the color developing reaction. All solutions were prepared with 0.1 mg of cholic acid (in 1 ml of distilled water), 6 ml of 16 N sulfuric acid, and 1 ml of 1 per cent aqueous furfural reagent. Reaction temperature 65° , reaction time 13 minutes. Curve A, undiluted solution; Curve B, solution diluted with 5 ml of glacial acetic acid; Curve C, solution diluted with 5 ml of 95 per cent ethyl alcohol.

gated (cholic acid) or conjugated (glycocholic and taurocholic acids). Of the compounds likely to occur in bile and blood, only tryptophane and other indole derivatives react with furfural with the production of pigments with appreciable absorption in the region of the spectrum where cholate is determined. However, filtrates and extracts of bile and blood, prepared as described, contain only traces of indoles as indicated by application of the quantitative method of Allsopp (20). This can be used in doubtful cases. Certain special aspects of the question of specificity of the method as applied to the determination of cholate in blood are considered in subsequent sections of this paper.

Preparation of Bile Filtrates for Cholate Determinations—Reinhold and Wilson (1) recommended the use of alcohol for the deproteinization of bile for cholate determinations. Doubilet (4) suggested the use of zinc sulfate and sodium hydroxide solutions. Both of these procedures were examined for their suitability for photometric analyses, and both were found to be satisfactory under specified conditions. Removal of protein with alcohol is simple and rapid, but the filtrates are contaminated with bile pigments. When the ratio of cholate to bile pigment is high, the presence of pigment causes only slight error if blank corrections are made with the filtrates. The error becomes significant when the ratio of cholate to pigment is low, and the zinc method is preferable for such samples.

When alcoholic filtrates are used, aliquots of the alcoholic solutions are evaporated in test-tubes by immersion in a bath of boiling water. The residue is dissolved in 1 ml of distilled water, color is developed as described in a preceding section, and the extinction at $620\text{ m}\mu$, ϵ'_1 , is calculated from the transmittance. The transmittance of a reagent blank is taken as unity. When the filtrates contain appreciable amounts of bile pigments, corrections are made with filtrate blanks. This blank is prepared from a mixture of the following composition: the dried residue from an equivalent portion of alcoholic bile filtrate, 2 ml of distilled water, and 6 ml of 16 N sulfuric acid. This solution is heated for 13 minutes at 65° . At the end of the period of heating, the solution is cooled to room temperature and is diluted with 5 ml of glacial acetic acid. The extinction, ϵ'_2 , is calculated from the fractional transmittance based on that of a reagent blank as unity. The amount of cholic acid in the aliquot of the alcoholic filtrate is calculated from the equation, $C = (\epsilon'_1 - \epsilon'_2)/\epsilon_s$ (see foot-note 2).

The cholic acid concentration can be calculated also from the extinction at $660\text{ m}\mu$. If close agreement is not obtained between the values calculated from measurements at 620 and $660\text{ m}\mu$, the discrepancy suggests that extraneous substances in the bile filtrates are interfering with the determinations. The values calculated from measurements made at $620\text{ m}\mu$ are usually more accurate than those based on determinations at $660\text{ m}\mu$ for reasons explained in a preceding section.

Filtrates of bile prepared by a method similar to that of Doubilet (4) are almost colorless and filtrate blanks are negligible. Color development is carried out directly on 1 ml portions of these aqueous filtrates or appropriate dilutions thereof.

Typical analyses of human, ox, and canine bile are presented in Table II. Both methods for the preparation of protein-free filtrates were employed for comparison, and recovery determinations were made after addition of known amounts of cholate, glycocholate, and taurocholate salts to other samples of the same bile specimens.

Procedures for Determination of Cholate in Whole Blood and Plasma—The determination of cholate in blood has been beset by two principal difficulties, namely, uncertainties regarding the specificity of the photometric methods and problems encountered in the attempts to prepare colorless, protein-free filtrates of blood without analytically prohibitive losses of cholate by precipitation and by adsorption on the protein residues. The latter difficulty has been minimized by several investigators (3, 14, 21) by

TABLE II

Determination of Cholate in Typical Samples of Bile and Analytical Recovery of Cholate, Glycocholate, and Taurocholate Salts Added to Bile

The analytical figures are averages of six determinations. The following symbols are used: C = cholate, Gc = glycocholate, Tc = taurocholate. The statistical notations used in this and the following tables are as follows: a = average error of a single determination (see Table I), A_1 = the average error of the average analytical recovery. A_2 is calculated from the following equation: $A_2 = \pm \sqrt{A_1^2 + A^2}$, in which A_1 and A are the average errors of the average initial and final concentrations, respectively. $A = \pm \Sigma v/n\sqrt{n}$. Values for A_1 and A_2 are omitted from the table

Type of bile	Method for removing protein	Initial concentration of cholate in bile (average)	$a \pm$	Type of bile salt added	Amount of bile salt added per liter	Final concentration of cholate in bile (average)	Analytical recovery of added bile salt (average)	$A_2 \pm$
		mm	per cent		mm	mm	per cent	per cent
Gallbladder, ox	Alcoholic	132	2.8	C	100	230	98.0	2.7
	Zinc	129	2.3	"	100	227	98.0	2.4
	"	129	2.3	Gc	100	225	96.0	2.3
	"	129	2.3	"	150	275	97.5	1.8
	"	129	2.3	Tc	100	223	94.0	2.5
Gallbladder, man	Alcoholic	112	2.6	C	100	207	95.0	2.4
	Zinc	110	2.5	"	100	206	96.0	2.2
Gallbladder, dog	Alcoholic	152	2.6	Gc	100	248	96.0	2.8
	Zinc	150	2.1	"	100	248	98.0	2.5
Hepatic, dog	Alcoholic	37	3.0	Tc	100	130	93.0	1.4
	Zinc	38	2.4	"	100	133	95.0	1.3
	"	38	2.4	"	150	179	94.0	1.3
	"	38	2.4	Gc	150	186	98.5	1.2

precipitation of proteins with alcohol and removal of pigments by addition of barium hydroxide.

We have used two methods for the preparation of filtrates and extracts of blood for cholate estimations. The first is a procedure which is suitable for routine analyses because of its relative simplicity; it is an adaptation of the method of Josephson (3). Because of the difficulty with Josephson's technique of removing lipids from the filtrates by extraction with ethyl acetate, we have substituted ethyl ether at that stage. This substitution

renders centrifugation unnecessary. According to Josephson's procedure, the alcoholic filtrates are evaporated by boiling in open tubes. This operation is tedious, and it is a source of difficulty because of partial hydrolysis of lipids, leading to turbidity at a later stage, and because of partial destruction of cholate. This difficulty was eliminated by evaporation of the filtrates *in vacuo*. Addition of glacial acetic acid to the final solutions after color development is also an improvement in the method, because it aids in the elimination of turbidity. The second method which we are presenting is an extended procedure which provides greater specificity.

Short Method for Routine Determination of Cholate in Blood and Plasma (Modification of Josephson's Procedure)—Into a 250 ml volumetric flask containing 50 ml of absolute ethyl alcohol (absolute alcohol is used to minimize water in the final solution) a 5 ml portion of barium hydroxide reagent³ is introduced. During continuous agitation of the flask, exactly 10 ml of heparinized⁴ whole blood or plasma are added slowly, and the flask is immersed in boiling water for 5 minutes. After the period of heating, absolute alcohol (neutralized to litmus) is added almost to the 250 ml mark, and the flask is kept at room temperature for about 12 hours. The long period is necessary for complete precipitation of pigments. Then the volume is adjusted exactly to 250 ml, and the solution is filtered (Whatman filter paper, No. 42). If the first portion of the filtrate is turbid, it is passed through the paper again. No attempt is made to collect more than about 210 ml of the filtrate, and the precipitate is not washed.⁵

Excess barium is precipitated from the alcoholic filtrate by addition of a drop of concentrated sulfuric acid. After flocculation of the barium sulfate, the solution is made alkaline to litmus again by the addition of 2 or 3 drops of a concentrated solution of sodium hydroxide. If only a few drops of sulfuric acid and sodium hydroxide solutions are used, the slight dilution error is neglected. The solution is permitted to stand at room temperature for several hours to allow barium sulfate and sodium sulfate to settle completely. At the end of this period, it is filtered through Whatman No. 42

³ This reagent is a saturated (room temperature) aqueous solution of barium hydroxide containing 1 gm of barium acetate per 100 ml.

⁴ Whole blood and plasma containing oxalate or citrate should not be used, since these anions cause precipitation of barium.

⁵ Theoretically, a requisite condition for omission of the technique of washing the precipitate would be equal distribution of cholate between equal volumes of solution and of wet precipitate. Actually, the distribution coefficient is slightly less than unity. Nevertheless, the washing technique was not adopted, inasmuch as comparative experiments and analyses demonstrated that it caused technical complications and disproportionate extraction of interfering substances which offset the theoretical advantage. Recovery analyses are sufficiently consistent to permit the use of factors to correct for over all losses of cholate. However, all analyses recorded in our tables are uncorrected.

paper The precipitate is not washed The filtrates usually are clear and colorless, but occasionally they become slightly cloudy on standing, owing to additional precipitation of sodium sulfate Such turbidity can be overcome by another filtration, but this is not essential, since the sodium sulfate precipitate does not interfere with subsequent phases of the analysis The rare samples which are colored at this stage are discarded, and the analysis is repeated

Exactly 200 ml of the filtrate are evaporated to a small volume in several stages in a 50 ml flask attached by means of a ground glass joint to a vacuum distilling head⁶ During the vacuum distillation of the alcohol, the temperature of the bath in which the flask is immersed is maintained at or below 35° When the entire 200 ml portion of filtrate has been concentrated to about 10 ml, the concentrated solution is filtered to remove the precipitate which forms during evaporation The filtrate is collected in a special calibrated tube⁶ and the flask, funnel, and residue are washed several times with small portions of absolute alcohol in order that the transfer may be quantitative The total volume of solution in the tube should not exceed 15 to 20 ml The tube is attached to the distilling head with the capillary tube inserted to prevent bumping, and the alcohol is evaporated to dryness under reduced pressure with a bath temperature of 35°, or less

The alcohol-free residue is dissolved in 3 ml of 0.1 N sodium hydroxide solution, approximately 10 ml of peroxide-free ethyl ether⁷ (saturated with distilled water) are added and the tube is closed with a ground glass stopper and shaken for several minutes The ether layer is permitted to separate and is removed by means of a suction tube The extraction with ether is repeated three additional times to complete the removal of cholesterol and neutral fat The ether fractions are combined and are extracted once with about 1 ml of distilled water which is made alkaline with a few drops of dilute sodium hydroxide solution This washing is added to the principal aqueous fraction in the tube, and residual ether is removed from the solution by immersing the tube in a bath of hot water for a few minutes The solution is cooled and is neutralized to litmus The volume is adjusted exactly

⁶ A suitable vacuum distilling apparatus with interchangeable ground glass joints can be obtained from the Scientific Glass Apparatus Company, Bloomfield, New Jersey (catalogue No J 1447) For the final evaporation of the filtrate, we use a special calibrated tube similar to the one described by Josephson (3), but provided with a ground joint for attachment to the vacuum distilling head

⁷ Dr R M Forbes of Wayne University College of Medicine has observed that some samples of c.p. ethyl ether contain peroxides in amounts which are sufficient to interfere with the determination of cholate perhaps by oxidation of the bile acid Before use in this method, all samples of ethyl ether should be tested (22) to insure freedom from peroxides Peroxides can be removed from ether by the method of Taylor and Smith (23)

to the 5 ml mark in the constricted portion of the tube. For the sake of clarity in subsequent discussion, this solution is designated Solution S.

1 ml portions of Solution S are used for color development by the procedure described in the section on extinction-concentration relationships.

TABLE III

Concentrations of Cholate in Whole Blood and Plasma from Peripheral Circulation of Normal Animals and Analytical Recoveries of Cholate Salts Added to These Blood Samples

All analyses reported in this table were made on filtrates prepared by the short, routine method. The analytical figures are the averages of four determinations. The statistical figures are the average errors of the averages. C = cholate, Gc = glycocholate, Tc = taurocholate.

Animal and type of blood sample	Initial concentration of cholate (as cholic acid)	Type of bile salt added	Amount of bile salt added (as cholic acid)	Final concentration of cholate (as cholic acid)	Analytical recovery of added cholate
	mg per 100 ml		mg per 100 ml	mg per 100 ml	per cent
Hog plasma	0.2 ± 0.1	C	20	18.8 ± 0.8	93 ± 4
" "	0.2 ± 0.1	Gc	20	19.4 ± 0.8	96 ± 4
" "	0.3 ± 0.1	C	15	14.4 ± 0.6	94 ± 4
" "	0.2 ± 0.1	Gc	15	14.0 ± 0.7	92 ± 5
" "	0.2 ± 0.1	Tc	15	13.4 ± 0.9	88 ± 6
" "	0.2 ± 0.1	C	10	8.9 ± 0.5	87 ± 5
" "	0.2 ± 0.1	Gc	10	8.6 ± 0.5	84 ± 5
" "	0.2 ± 0.1	C	5	4.5 ± 0.3	86 ± 6
" "	0.2 ± 0.1	Tc	5	4.3 ± 0.4	82 ± 8
" "	0.2 ± 0.1	C	4	3.5 ± 0.3	83 ± 8
" "	0.2 ± 0.1	Gc	4	3.3 ± 0.3	78 ± 8
" "	0.2 ± 0.1	"	3	2.6 ± 0.3	80 ± 11
" "	0.2 ± 0.1	"	2	1.7 ± 0.2	75 ± 11
" "	0.2 ± 0.1	Tc	1	0.8 ± 0.2	60 ± 22
" "	0.2 ± 0.1	C	0.5	0.5 ± 0.2	60 ± 44
" whole blood	0.3 ± 0.1	"	20	17.9 ± 0.9	88 ± 5
" " "	0.4 ± 0.2	"	10	8.8 ± 0.5	84 ± 6
" " "	0.4 ± 0.2	Gc	"	1.8 ± 0.2	70 ± 14
Ox " "	0.6 ± 0.2	C	5	4.6 ± 0.4	80 ± 9
" " "	0.6 ± 0.2	Gc	10	9.3 ± 0.5	87 ± 5
" plasma	0.5 ± 0.2	"	5	4.8 ± 0.3	86 ± 7
Dog whole blood	0.7 ± 0.2	Tc	10	9.0 ± 0.4	83 ± 4
Human " "	0.5 ± 0.2	Gc	10	9.0 ± 0.5	85 ± 5
" plasma	0.4 ± 0.2	"	5	4.5 ± 0.3	82 ± 7

The extinction, ϵ'_1 , is calculated from the fractional transmittance with the transmittance of a reagent blank as unity. Correction for slight turbidity or a trace of extraneous color in Solution S is made from the extinction, ϵ'_2 , of a filtrate blank, as described in the section on bile analyses. The

concentration of cholic acid in Solution S (mg per ml) is multiplied by the factor 62.5 to obtain the concentration of cholate in the blood or plasma in mg per 100 ml if the exact volumes of samples and filtrates used in the determination are those specified above. For the analysis of specimens of blood or plasma with concentrations of cholate less than 2 mg per 100 ml, samples larger than 10 ml must be used, and the volumes of alcohol and barium hydroxide reagent are increased proportionally. Applications of the routine method are recorded in Tables III and IV.

Extended Method for Preparation of Extracts of Blood for Cholate Determinations—Preliminary extraction of the blood or plasma sample is conducted as described in the preceding section with the exception that the final aqueous solution, S, is not neutralized and the volume is not adjusted

TABLE IV

Cholate Concentrations in Blood from Peripheral Circulation of Dogs with Common Bile Duct Obstructions before and after Oral Administration of Dried Ox Bile

All analyses were performed on blood filtrates prepared by the short, routine method

No of experiments	Duration of obstruction	Average initial concentration of blood cholate (as cholic acid)	Dried ox bile administered orally	Interval between bile administration and blood collection	Average final concentration of blood cholate (as cholic acid)
	days	mg per 100 ml	gm	hrs	mg per 100 ml
2	1	6.3	2	5	14.2
2	2	7.6	2	5	17.4
1	2	9.2	3	5	24.1
3	4	4.2	2	3	10.1
2	10	2.9	3	6	13.2
2	20	1.5	3	5	8.7

to 5 ml. This solution is transferred quantitatively to a Pyrex test-tube which has been constricted near the neck. The total volume of solution plus aqueous washings is about 6 ml. Ethyl alcohol and solid sodium hydroxide are added in such amounts that the final solution contains about 40 per cent alcohol and 12 per cent sodium hydroxide. The tube is sealed with a flame at a point in the constricted portion, which will leave less than 2 ml of space above the surface of the solution. During sealing most of the air is driven from the tube by alcohol vapor. After the sodium hydroxide has dissolved completely, the tube is heated in an autoclave at 115–120° for 10 hours to hydrolyze the conjugated bile salts. At the end of the period of hydrolysis, the neck of the tube is broken, and the solution is transferred quantitatively to a flask and evaporated to a small volume under reduced pressure to remove alcohol. The residue is transferred quantitatively with

water to a separatory funnel, and the solution is acidified to about pH 1 with sulfuric acid while the funnel is cooled in a bath of ice water (Transfer to the separatory funnel is carried out before acidification in order to prevent loss of cholic acid by precipitation) After five extractions of the acidified solution with 15 ml portions of peroxide-free ethyl ether, the aqueous phase is discarded, and the combined ether extracts are washed once with a 5 ml portion of 0.1 N sulfuric acid. The washing is discarded

TABLE V

Hydrolysis of Conjugated Cholates in Alkaline Solutions

In each experiment the total volume of solution was 10 ml. All figures are averages of five separate experiments

Type of conjugated bile salt	Cholic acid equivalent	Conditions for hydrolysis					Analytical recovery of cholic acid
		Apparatus used	Temperature	Concentration of ethyl alcohol	Concentration of sodium hydroxide	Duration of hydrolysis	
	mg		C	per cent	per cent	hrs	mg
Glycocholate	1.0	Flask with reflux condenser	80-90	50	7.5	6	0.5 ± 0.1
	2.5		80-90	50	7.5	6	1.15 ± 0.3
	1.0		80-90	50	7.5	12	0.71 ± 0.1
	1.0		80-90	50	15	12	0.83 ± 0.1
	1.0		80-90	50	15	15	0.89 ± 0.1
	1.0	Sealed tube in autoclave	100	50	15	5	0.62 ± 0.2
	1.0		100	50	15	15	0.91 ± 0.1
	1.0		115-120	50	15	5	0.74 ± 0.2
	1.0		115-120	50	15	10	0.96 ± 0.06
	1.0		115-120	40	12	10	0.98 ± 0.02
	2.0		115-120	40	12	10	1.98 ± 0.05
	5.0		115-120	40	12	10	4.95 ± 0.10
Taurocholate	1.0		115-120	40	12	10	0.94 ± 0.03
	2.0		115-120	40	12	10	1.92 ± 0.05
	1.0		115-120	50	15	10	0.92 ± 0.04
	1.0		115-120	50	15	15	0.96 ± 0.03

Then the ether fraction is extracted four times with 5 ml portions of 6.7 N hydrochloric acid (saturated with ether prior to use in the extraction). Cholic acid is extracted from ether by hydrochloric acid solutions of this concentration (24) (see Table VI). The combined acid extracts are placed in a separatory funnel, and the mixture is adjusted to about pH 1 by addition of a 20 per cent solution of sodium hydroxide. After five extractions with 15 ml portions of peroxide-free ethyl ether, the ether extracts are combined and are washed once with 5 ml of 0.1 N sulfuric acid. The aqueous phases are discarded. The ether fraction is made slightly alkaline and

is evaporated in several stages in the special tube and distilling apparatus described in the preceding section (see foot-note 6) The ether-free residue is dissolved in distilled water, the solution is made alkaline to litmus with sodium hydroxide, and the volume is adjusted exactly to the 5 ml mark in the constricted portion of the tube (The final aqueous solution may be brought accurately to a smaller volume if a preliminary analysis has indi-

TABLE VI

Distribution of Cholic Acid between Ethyl Ether and Solutions of Hydrochloric and Sulfuric Acids

In these experiments various quantities of cholic acid were dissolved in 25 ml portions of ethyl ether and extracted with 5 ml volumes of a variety of solutions of mineral acids. The mineral acid solutions were saturated with ethyl ether before use in the extractions. However, the recorded concentrations of these solutions are those which existed before saturation with ether.

Total quantity of cholic acid	Type and concentration of mineral acid	Average distribution of cholic acid at equilibrium		
		Concentration in mineral acid (A)	Concentration in ether (E)	Distribution ratio (A/E)
mg		mg per 100 ml	mg per 100 ml	
1	0.5 N H ₂ SO ₄	0.60	3.63	0.16
1	2.0 " "	1.0	3.60	0.28
1	5.0 " "	1.3	3.56	0.36
1	6.0 " "	1.4	3.48	0.40
1	7.0 " "	1.8	3.42	0.53
1	8.0 " "	2.6	3.36	0.77
2	8.0 " "	4.8	6.68	0.72
1	1.7 " HCl	1.8	3.54	0.51
1	3.4 " "	3.6	3.14	1.15
1	4.9 " "	10.0	2.00	5.0
1	6.0 " "	14.5	1.06	13.7
1	6.7 " "	16.0	0.64	25.0
0.5	6.7 " "	8.1	0.34	23.8
0.1	6.7 " "	1.7	0.07	24.2
2.0	6.7 " "	32.4	1.22	26.6

cated that the concentration of cholate is low) Cholate is determined in 1 ml samples of this solution, as described in the preceding section.

In the development of this extended method for the preparation of extracts of blood and plasma for cholate determinations, several investigations were necessary to establish satisfactory conditions. By alkaline hydrolysis of Solution S the conjugated cholates (glycocholate and taurocholate) are converted to cholate, thus permitting extraction of cholic acid with ethyl ether after acidification of the hydrolysate. (Unconjugated bile acids are soluble in ethyl ether, but conjugated bile acids are practically insoluble in

that solvent) This procedure eliminates those substances which are preferentially soluble in the acid aqueous phase Numerous conditions for

TABLE VII

Concentrations of Cholate in Whole Blood and Plasma from Peripheral Circulation of Normal Animals and Analytical Recoveries of Cholates Added to These Samples

All analyses reported in this table were performed on blood extracts prepared by the extended method The analytical figures are the averages of four determinations The statistical figures are the average errors of the averages C = cholate, Ge = glycocholate, Tc = taurocholate

Animal and type of blood sample	Initial concentration of cholate (as cholic acid)	Type of bile salt added	Amount of bile salt added (in terms of cholic acid)	Final concentration of cholate (in terms of cholic acid)	Analytical recovery of added cholate
	mg per 100 ml		mg per 100 ml	mg per 100 ml	per cent
Hog plasma	0.1 ± 0.06	C	20	17.9 ± 1.0	89 ± 5
" "	0.1 ± 0.07	"	15	12.5 ± 0.9	83 ± 6
" "	0.05 ± 0.04	Ge	5	3.9 ± 0.4	77 ± 8
" whole blood	0.15 ± 0.05	"	15	12.2 ± 1.0	80 ± 7
" " "	0.15 ± 0.05	Tc	10	7.9 ± 0.7	78 ± 7
Ox " "	0.3 ± 0.1	Ge	10	8.5 ± 0.7	82 ± 7
Dog " "	0.5 ± 0.2	"	5	4.2 ± 0.4	74 ± 9
whole blood	0.4 ± 0.2	"	10	8.1 ± 0.6	77 ± 6

TABLE VIII

Cholate Concentrations in Blood from Peripheral Circulation of Dogs and Men with Common Bile Duct Obstructions before and after Oral Administration of Dried Ox Bile

All analyses reported in this table were made on blood extracts prepared by the extended method, with the exception of the analyses of blood from the human cases The latter were performed on blood filtrates which were rendered protein free by the use of zinc sulfate and sodium hydroxide

Animal	Duration of obstruction	Initial concentration of cholate (as cholic acid)	Dried ox bile administered orally	Interval between bile administration and blood collection	Final concentration of cholate (as cholic acid)
	days	mg per 100 ml	gm	hrs	mg per 100 ml
Dog	1	4.2	2	5	10.6
"	2	8.4	2	5	15.1
"	21	2.3	2	5	9.4
Man	?	1.6	3	15	3.7
"	?	1.1	5	10	7.4
"	?	9.2			

hydrolysis were tried for the selection of an optimum which would yield maximum hydrolysis of conjugated cholates with minimum destruction of cholate These experiments are summarized in Table V

The ether extracts of the acidified hydrolysates contain, in addition to cholic acid, small amounts of fatty acids and other ether-soluble impurities. Further fractionation is accomplished by extraction of the ether phase with concentrated solutions of hydrochloric acid. Wieland and Seibert (24) reported that bile acids can be extracted from ether by such a procedure, thus distinguishing them from many other ether-soluble organic acids. However, they applied the technique only to the isolation of relatively large quantities of bile acids from bile, and they did not record extensive studies of the distribution. Experiments to determine optimum conditions for the extraction of small quantities of cholic acid from ether are summarized in Table VI. A 6.7% solution of hydrochloric acid was adopted for the analytical procedure, inasmuch as this concentration provides efficient extraction of cholic acid with apparently minimum extraction of extraneous material. Hydrochloric acid solutions are much more effective for extracting cholic acid from ether than are solutions of sulfuric acid of corresponding concentrations (Table VI).

Cholate was determined by the routine method and by the extended procedure in blood from the peripheral circulation of normal animals (Tables III and VII) and of those with biliary obstructions (Tables IV and VIII). In addition, Tables III and VII include data from recovery analyses of samples to which known amounts of cholate, glycocholate, and taurocholate salts were added.

DISCUSSION

The fact that cholate does not occur in the *bile* of the hog provides substantial basis for the belief that cholate is not present in the *blood* of this animal. Therefore, the absence (except traces) of material yielding positive color reactions for cholate in extracts of hog blood prepared by the short, routine method and by the extended procedure is an important indication of the specificity of the methods. The traces of "cholate" determined in hog blood (Tables III and VII) represent errors resulting from slight absorption at 620 $m\mu$ by extraneous substances for which the blanks do not provide completely adequate correction. Extracts prepared by the extended method (Table VII) contain smaller amounts of interfering substances than those prepared by the routine procedure (Table III).

The data of Tables III and VII demonstrate that the accuracy of analytical recovery diminishes with decreasing concentrations of cholate in blood. Analytical recoveries of cholate from serum and plasma samples are somewhat greater than recoveries obtained from whole blood specimens, and extract blank corrections are smaller. Recoveries with the extended method are slightly lower than those with the routine procedure, but the former provides greater specificity.

Analyses of blood samples collected from the peripheral circulations

(limb veins) of various normal animals (Tables III and VII) demonstrated that cholate was not present in concentrations which could be determined accurately by these methods (the concentrations generally were less than 1 mg per 100 ml of blood). However, it is of interest that the analytical figures for concentration of blood cholate in animals which normally secrete cholate in their bile (ox, dog, and man) were greater than those for the hog, which does not secrete cholate. This provides additional basis for the belief that small amounts of cholate actually are present in the systemic blood of these three animals. We have not studied by these methods a sufficiently large series of normal animals to establish statistically the upper limits of the normal ranges of blood cholate concentrations. In contrast to the normal condition, blood samples from limb veins of men and dogs with biliary obstructions were found to contain cholate in analytically determinable and appreciable amounts except in those cases in which the obstruction was of long duration and in which there was reason to expect some liver damage with consequent failure of the synthesis of cholate (Tables IV and VIII). Oral administration of cholates (sodium salts of cholic, glycocholic, and taurocholic acids), or of desiccated ox bile containing cholates, to animals with biliary obstructions increased the concentration of cholate in the peripheral circulation (Tables IV and VIII). These observations indicate that normally the cholates remain largely in the enterohepatic circulation without passing the liver and entering the peripheral circulation in appreciable amounts unless the pathway for the excretion of bile is blocked.

SUMMARY

The method of Reinhold and Wilson has been adapted for the determination of cholates with a filter photometer. With this modified procedure, the average analytical recovery of cholate is 99.1 ± 0.7 per cent of the theoretical for pure solutions and 96 ± 2.1 per cent for bile. The average errors of single determinations are ± 1.5 per cent for pure solutions of cholates and ± 2.5 per cent for bile.

Two methods are described for preparing extracts of blood for cholate analyses, one, a modification of Josephson's procedure, is suitable for routine determinations, and the other is an extended method which provides greater specificity. With the routine procedure, analytical recoveries of cholate added to samples of blood are 92 ± 5 per cent of the theoretical for concentrations between 15 and 20 mg per 100 ml and 85 ± 6 per cent for concentrations of 5 to 10 mg per 100 ml of blood. Smaller concentrations are estimated with less accuracy, and only rough approximations are obtained for concentrations below 1 mg per 100 ml. Determinations of cholates in blood collected from limb veins of men and dogs

with biliary obstructions demonstrated appreciable concentrations, particularly after oral administration of these bile salts. By contrast, in normal animals cholate concentrations generally were found to be less than 1 mg per 100 ml of blood.

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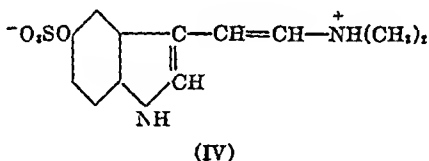
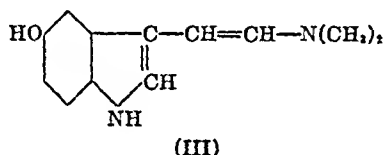
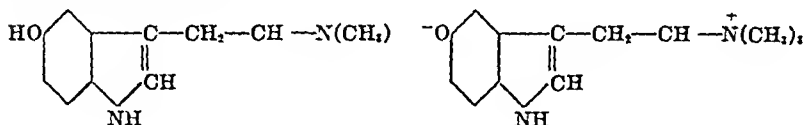
THE BASIC CONSTITUENTS OF THE VENOM OF SOME SOUTH AMERICAN TOADS

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In the venom of the toads there is always present a group of substances designated the basic constituents (1). They are adrenalin and the indole derivatives, bufotenine (I), bufotenidine (II), dehydrobufotenine (III), and bufothiionine (IV).



In the work described in this paper these compounds have been investigated as components of the venom of some South American toads. New information is given on the venom of *Bufo chilensis*, *B. crucifer*, and *B. spinulosus* and some complementary data on *B. arenarum* and *B. paracnemis*.

EXPERIMENTAL

Methods—In some experiments dried venom was employed, in others the venom was expressed from the skins and dried *in vacuo*. The methods employed in each case are slightly different.

Dried Venom—The method described by Wieland, Hesse, and Huttel (2) was followed with some modifications. The dried venom is exhaustively

extracted with chloroform which dissolves the neutral constituents (bufagins) The residual venom is then dried and twice extracted with large amounts of cold methanol The solution is evaporated at a low temperature, the residue is dissolved in a small amount of ethanol, and chloroform is added until no more precipitation takes place The solution contains the toxic bufotoxins, the basic constituents are in the precipitate This precipitate is well dried and dissolved in water The faintly acid solution is filtered, brought to pH 2.5, and extracted with ether (In all operations peroxide-free ether must be employed) By evaporation of the ether, suberic acid is obtained from some venoms

The residual water solution is now made alkaline with barium hydroxide and again extracted with ether, from which bufotenine is obtained as a yellow syrup This syrup is dissolved in a small amount of hydrochloric acid and the solution divided into two parts To one flavianic acid is added, and, by heating and then cooling, the bufotenine flavianate crystallizes as red needles, which can be purified by recrystallization from water, m p 131–133° (3) To the other solution picric acid is added, the resulting red bufotenine dipicrate, m p 174°, can be transformed into the yellow monopicrate, m p 180°, by boiling with benzene and recrystallizing from water (4) The bufotenine can be further characterized by transformation into bufotenidine hydroiodide, m p 215°, by treatment with methyl iodide (5)

The alkaline water solution is freed of barium by addition of dilute sulfuric acid and tested for bufotenidine by addition of flavianic acid None of the samples contained this base The solution is then concentrated at low temperature and about pH 7 From time to time, usually when the volume is reduced to one-half, a sample is taken, made slightly acid, and treated with picric acid If dehydrobufotenine is present, a yellow picrate is obtained, which after recrystallization from absolute ethanol melts at 187° From 50 per cent ethanol it melts indefinitely at 147–150° Wieland and Wieland (4) point out that the low melting form is transformed into the high melting by boiling with mineral acids

The dried venom residue which has been extracted with chloroform and with methanol can be further employed for the isolation of adrenalin by the method described by Deulofeu (6) after extraction with a 1 per cent water solution of acetic acid

By this method all the above basic constituents of a toad's venom can readily be characterized We have been unable to obtain bufotoxinone, which can be very easily isolated from dried toad skins

Toad Skins—The well dried toad skins were extracted twice with large volumes of 70 to 80 per cent ethanol The united extracts are concentrated at low temperature almost to dryness and the residue treated with

several portions of petroleum ether, until this solvent leaves no residue on evaporation

The residue, insoluble in petroleum ether, is then treated with a small amount of absolute ethanol, when most of it dissolves, leaving insoluble inorganic salts and bufothionine. This insoluble residue is filtered and by crystallization from water, with the aid of charcoal if necessary, the bufothionine can be isolated as colorless prisms, m p 250° (darkening from about 240°), and further characterized by transformation into dehydrobufotenine hydrochloride (m p 244°) by boiling with 2 N hydrochloric acid

The ethanolic solutions are then concentrated to a small volume and poured into a large amount of iced water. A precipitate, mainly bufagins, is removed, the filtrate containing the bases is concentrated at low temperature, filtered again if some precipitate appears, and brought to pH 2.5. From it suberic acid, bufotenine, dehydrobufotenine, and even bufotendine, if present, can be isolated by the method described for the dried venom

Adrenalin could not be isolated, even from skins of toads of which the dried venom contained it. It is apparently destroyed by the process.

In all cases mixed melting points with authentic preparations were determined.

Results

Bufo chilensis—This is the most common toad in Chile. From skin bufothionine, m p 252° , was isolated and characterized by transformation into dehydrobufotenine hydrochloride, m p 243° . From the alcoholic extract suberic acid melting at 143° was isolated. Further, bufotenine was characterized as its flavanate, m p $130-131^{\circ}$, and its red dipicrate, m p $172-173^{\circ}$, which on boiling with benzene passes into the yellow monopicate, m p 179° . Dehydrobufotenine was isolated from the solution, after separation of the bufotenine, as the yellow picrate, m p 187° .

Bufo crucifer—This is a small toad from Brazil. Only 350 dried skins were available.

Bufothionine was characterized by its melting point (250°) and transformation into dehydrobufotenine hydrochloride (m p $240-241^{\circ}$). Bufotenine (picrate m p 173° and flavanate m p $130-131^{\circ}$) and dehydrobufotenine (picrate m p 187°) were found among the ethanol-soluble substances.

Bufo spinulosus—This is a common species of toad in Peru. Only bufothionine (m p 249°) and dehydrobufotenine (yellow picrate m p 187°) could be characterized. As the amount of material available was small (forty-three dried skins), the possibility that bufotenine or bufotendine was present is not excluded.

Bufo paracnemis—This large toad from northern Argentina was studied by Deulofeu and Mendive (3) who isolated bufotenine and adrenalin from the dried venom. Working with the dried skin, we have been able to characterize bufothionine, m p 249°, which with boiling 2 N hydrochloric acid yielded dehydrobufotenine hydrochloride, m p 240–241°. Bufotenine was isolated as the red dipicrate, m p 174°, from which the base was regenerated and converted (by treatment with methyl iodide) into bufotenidine hydroiodide, m p 213–215°. Dehydrobufotenine (yellow picrate m p 187°) was also present, but adrenalin could not be characterized in the residual liquors.

Bufo arenarum—This is the predominant species in the central part of Argentina. In the dried venom bufotenine and dehydrobufotenine were found by Chen, Jensen, and Chen (7), adrenalin by Deulofeu (6), and bufothionine by Jensen (8). From the skins Wieland, Konz, and Mittasch (5) isolated bufothionine, bufotenine, and dehydrobufotenine. We have confirmed the presence of these bases in dried venom and dried skins with the exception that adrenalin could not be obtained from the skins.

DISCUSSION

The data here presented supplement the available information on another South American toad, *Bufo marinus*, in the secretion of which Abel and Macht (9) first characterized adrenalin. Chen and Chen (10) later demonstrated dehydrobufotenine and Deulofeu and Mendive (3) bufotenine. No bufothionine has been reported in the secretion, but as the secretions of *B. marinus* and *B. paracnemis* have practically the same chemical constituents, including the bufagins (3), we feel that bufothionine would be found if a search were made in the dried skins.

The venoms of those South American toads are characterized by the presence in its basic fraction of dehydrobufotenine, of its conjugated sulfuric acid derivative bufothionine, and of its reduction product bufotenine. The mechanisms of the interconversion of those three substances in the toad organism are unknown, but it is noteworthy that bufotenine possesses a pressor activity which is lacking in dehydrobufotenine and bufothionine. Bufotenidine, found in many species of toads from other regions, could not be detected in the toads which we examined. Its absence is especially certain in the cases of *Bufo paracnemis* and *B. arenarum*, of which we had large amounts at our disposal, and of *B. marinus*, a specimen of the dried venom of which was examined solely for bufotenidine.

SUMMARY

1. The basic constituents of the venom of the South American toads *Bufo chilensis*, *B. crucifer*, *B. spinulosus*, *B. paracnemis*, and *B. arenarum* have been investigated.

2 Bufothionine, bufotenine, and dehydrobufotenine are the most commonly present substances of this type. Adrenalin is always present in the dried venom. Bufotenidine has not been found in the dried secretions nor in the dried skins.

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EXPERIMENTS ON THE ACTIVATION OF FICIN

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At the present time a relatively large number of enzymes are known which have the property of being activated by sulfhydryl compounds or by cyanide ions. A number of these enzymes have been inactivated by treatment with oxidizing agents, and then largely reactivated by the above activators. The majority of investigators have concluded from these and similar experiments that activators such as cysteine function as reducing agents. In the cases of certain of these enzymes, such as urease (1, 2) and papain (3) a number of studies suggest that thiol groups, actual or potential, may be necessary for the enzymic activity.

Bergmann (4) in considering this theory of activation has pointed out that oxidation-reduction studies are complicated by the presence in most preparations of natural activators, such as glutathione, which may undergo the reactions attributed to the enzymes. Irving, Fruton, and Bergmann (5) observed that the activation of papain and cathepsin by HCN was reversed when the latter reagent was removed by an evacuation procedure, and that, also, dialysis of these enzymes to remove natural activators results in a similar loss of activity. Chiefly from these findings and from the observation (6) that papain becomes inactive when precipitated from cyanide solution with isopropyl alcohol, Bergmann concludes (4) that activators such as H_2S , HCN, and cysteine function, not only as reducing agents, but as coenzymes, and that the activation of papain and cathepsin involves the formation of (dissociable) compounds between the activators and the enzymes.

Greenberg and Winnick (7) have suggested that atmospheric oxygen may have an important effect in activation studies by its tendency to inactivate (oxidize) such enzymes as papain under certain conditions. The present authors have investigated the nature of the activation process of a papain-like enzyme and the significance of atmospheric oxygen by experiments involving preliminary dialysis of the activated enzyme to remove completely activators *under both aerobic and strictly anaerobic conditions*. Also the effect on enzymic activity of the removal of H_2S and HCN at reduced pressures in an atmosphere of nitrogen was deter-

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mined The enzyme employed in the present study was crystalline ficin The papainase character of this enzyme has been established by Walti (8)

EXPERIMENTAL

Enzyme Preparation—The crystalline ficin was kindly supplied by Merck and Company, Inc Its activity, unchanged by recrystallization, was of the order of 1.3 to 1.6 units per mg of enzyme nitrogen in the presence of 0.005 M cysteine at pH 7.0, as measured by the casein non protein nitrogen method of Northrop and Kunitz (9) This activity is intermediate between that of crystalline trypsin (9) and chymotrypsin (10)

Method of Determining Ficin Activity—The activity of the ficin solution was determined by the Northrop and Kunitz method just cited, but with casein prepared by the method of Van Slyke and Baker (11) and adjusted in phosphate buffer to pH 7.0 This method of assay appears to give a true measure of the initial stages of proteolysis in that only the formation of non-protein nitrogen is measured Duplicate analyses made on 3 ml portions of the trichloroacetic acid filtrates, with the boric acid modification of the micro-Kjeldahl method, agreed to within 1 per cent Blank analyses were run to correct for traces of non-protein nitrogen in the reagents and in the enzyme solutions (when cysteine was present) From the nitrogen content of the ficin solutions, and with the aid of a standard curve based on activity measurements with a series of dilutions of a ficin solution, the micro-Kjeldahl titrations could be expressed directly as ficin activity units

An unfailling qualitative indication of protease activity was the change of the color of the substrate to a pronounced white during digestions

Apparatus for Anaerobic Dialysis—The apparatus resembled a 12 inch Liebig condenser, except that the inner tube consisted of the upper and lower sections of a 50 ml burette joined by an 8 inch section of cellophane tubing of 1.8 cm diameter Two inlet tubes sealed to the lower part of the outer jacket served for the introduction of oxygen-free, double distilled water and purified nitrogen The water and nitrogen escaped through a single outlet at the upper part of the outer jacket

The inner tube was filled with the enzyme solution, which was kept free of oxygen by a stream of nitrogen gas supplied through a 3 mm glass tube The latter extended to the bottom of the inner tube

The double distilled water which circulated through the dialyzer (outer jacket) was freed of air by bubbling purified nitrogen gas through the water before and during the dialysis Approximately 12 liters of water were passed through the dialyzer during the course of a complete dialysis experiment The nitrogen was purified by passage over red hot copper and cooled by bubbling through redistilled water The entire dialysis was carried out at a temperature of approximately 10°

In order to assay the enzyme solution during the dialysis it was necessary to add 1 ml portions to the casein substrate under anaerobic conditions. This was accomplished with the aid of a special test-tube which could be evacuated through a standard taper joint terminating in a stop-cock. 5 ml of the casein substrate were placed in this test-tube and the latter was thoroughly evacuated. The test-tube was then connected by a short piece of rubber tubing to the stop-cock of the lower burette section of the dialysis apparatus.

1 (± 0.05) ml of enzyme solution was drawn into the evacuated test-tube by proper manipulation of the two stop-cocks. The test-tube was immediately detached from the burette and maintained at 35.5° for 20 minutes as required in the Northrop and Kunitz method.

Results

The data in Table I show that there was no great decrease in protease activity when the ficin solution, previously activated by neutral 0.03 M cysteine, was thoroughly dialyzed against water in the complete absence of oxygen. The negative nitroprusside test given by the enzyme solution (Test 5) at the end of the dialysis indicates that the removal of activator was complete. Tests made by the authors show that a positive, though very weak, nitroprusside test is still given by cysteine at a concentration of 0.0001 M.

Test 6 refers to an experiment in which 0.1 ml of 0.00066 M KMnO_4 was added to 1 ml of activator-free ficin (Test 5) prior to the addition of the latter to the casein substrate. Since no activator was present, the action of the KMnO_4 can only be viewed as an oxidation of the enzyme itself. In Test 7, 1 ml of the preceding enzyme solution previously inactivated by KMnO_4 was treated with 0.1 ml of 0.33 M cysteine (pH 7.0). The cysteine restored 80 per cent of the enzymic activity.

The effect of aerobic dialysis on the activity of ficin, previously activated by cysteine, is shown in Table II. The enzyme lost about 96 per cent of its activity by the end of 23 hours, when the nitroprusside test was completely negative. Probably some of the cysteine was oxidized by oxygen of the dissolved air during the dialysis. In the assay of the enzyme, 2 ml samples of the ficin solution were first mixed with 1 ml of water in Tests 1 to 4, or with 1 ml of 0.1 M cysteine (pH 7.0), 0.2 M cyanide (pH 4.5), or 0.2 M H_2S (saturated at 0°) in Tests 5 to 9. 1 ml samples of the resulting solutions were then assayed in each case. Test 5 shows that the addition of cysteine restored most of the activity to the aerobically dialyzed ficin. Treatment with HCN (Test 6) and H_2S (Test 8) also resulted in reactivation, although the activity of the ficin was less than when cysteine was

TABLE I

Dialysis of Ficin in Absence of Oxygen

About 50 ml of a solution at pH 5.1 containing 0.101 mg of ficin nitrogen per ml, and 0.03 M in cysteine, were dialyzed under anaerobic conditions. At the specified time intervals, 1 ml portions of the enzyme solution were assayed.

Test No	Time of dialysis	Ficin units per mg enzyme nitrogen	Remarks
	<i>hrs</i>		
1	0	1.28	Enzyme solution gives strongly positive nitroprusside test
2	1	1.35	Dialysate gives positive nitroprusside test
3	5½	1.13	" " " " "
4	10	1.13	" " very weak nitroprusside test
5	23	1.03*	Both dialysate and enzyme solution give negative nitroprusside tests
6		0.06	Test 5 treated with KMnO_4
7		0.82	" 6 " " cysteine

* The reduction in activity at this point may be due, at least in part, to the fact that papainase solutions slowly lose activity on standing (12)

TABLE II

Dialysis of Ficin under Aerobic Conditions and Evacuation of Reactivated Solution

About 50 ml of solution at pH 6.0 containing 0.071 mg of ficin nitrogen per ml, and 0.03 M in cysteine, were dialyzed against redistilled water previously saturated with air. At specified time intervals, portions of the enzyme solution were removed for assay.

Test No	Time of dialysis	Ficin units per mg enzyme nitrogen	Remarks
	<i>hrs</i>		
1	0	1.64	Enzyme solution gives strongly positive nitroprusside test
2	5	1.13	Dialysate gives positive nitroprusside test
3	10	0.33	" " very weak nitroprusside test
4	23	0.06	Both dialysate and enzyme solution give negative nitroprusside tests
5		1.28	Test 4 made 0.03 M in cysteine
6		0.71	" 4 " 0.067 " " HCN (pH 4.5)
7		0.65	" 6 after evacuation in nitrogen atmosphere
8		0.36	" 4 made 0.067 M in H_2S
9		0.40	" 8 after evacuation in nitrogen atmosphere

employed. This latter finding is in agreement with the authors' (unpublished) observations with papain and with the findings of Irving, Fruton, and Bergmann (5) with papain.

Tests 7 and 9 were designed to test the effect on ficin of the removal of volatile activators. The apparatus described in Fig 1 of the paper by Irving, Fruton, and Bergmann was employed in these experiments to concentrate 1 ml portions of the activated enzyme at 35° in a stream of purified nitrogen at reduced pressure. The apparatus was modified by the use of the lower portion of a burette with a 3-way stop-cock instead of the inlet tube, *B*, in Fig 1 of the above paper. The burette contained 5 ml of casein substrate and 0.9 ml of water. Purified nitrogen had been previously bubbled through the 5.9 ml of solution to remove air. Following the concentration of the enzyme solution to a volume of 0.1 to 0.2 ml in about an hour, the casein solution was anaerobically drawn into the evacuation vessel (maintained at 35.5°). After 20 minutes the latter vessel was opened, and 5 ml of 10 per cent trichloroacetic acid added, as required in the Northrop and Kunitz method. The results in Table II (Tests 7 and 9) show that the removal of activators caused no significant changes in enzymic activity.

The lack of activity of the papain following the removal of HCN or H₂S by evacuation in the experiments of Irving, Fruton, and Bergmann may be due to the fact that these investigators apparently did not exclude air from their substrate and did not add their oxygen-free enzyme solution to the substrate under anaerobic conditions.

The fact that ficin retains its activity when completely freed of activators by either dialysis or evacuation under anaerobic conditions argues against the coenzyme theory of papainase activation proposed by Bergmann. On the other hand, the inactivation of ficin by oxygen or by permanganate (in the absence of activators) and the reactivation by HCN and sulphhydryl compounds are in agreement with the view that papainases are active in a reduced form and inactive when oxidized.

Bergmann (4), in observing that papain was inactive toward HCN in the absence of traces of sulphhydryl compounds, has postulated the existence of two inactive forms of papain, designated α -trypsinase and β -trypsinase. The α form could be converted by sulphhydryl compounds into the β form, and the latter was then capable of activation by combination with HCN or sulphhydryl compounds.

Test 6 in Table II indicates that ficin, dialyzed free of added cysteine, is activated by cyanide alone. Also unpublished experiments by the present authors indicate that both ficin and papain, dialyzed free of traces of natural activators under aerobic conditions, can be reactivated by HCN, and that added traces of cysteine (0.0006 M concentration) do not cause a significant further increase in proteolytic activity. These findings are not in accord with the theory that papainases can exist in two different inactive forms.

In examining the oxidation-reduction theory of papainase behavior, it appeared to us of interest to determine the potential of a mixture of equal parts of active and inactive ficin solution, and to compare this value with the corresponding potential of the cysteine-cystine couple. 5 ml of the dialyzed, active ficin solution described in Table I were mixed with a second 5 ml portion of this same solution which had been previously inactivated by bubbling air through it for an hour, followed by nitrogen gas to remove the air. The potential of the mixed solutions (pH 7.3) was +0.17 volt with respect to the normal calomel electrode. Next, a solution 0.01 M in both cysteine and cystine was prepared. The potential of this solution was -0.38 volt with respect to the normal calomel electrode. The fact that these two potentials differ by about half a volt supports the view that cysteine readily reduces the oxidized form of ficin. The value of the oxidation potential of oxygen supports the view that reduced ficin tends to be readily oxidized by oxygen gas.

SUMMARY

A solution of crystalline ficin has been shown to retain its activity when freed completely of cysteine by anaerobic dialysis, or when freed of HS or HCN by evacuation in an atmosphere of nitrogen. Aerobic dialysis of the activated enzyme resulted in virtually a complete loss of activity, which, however, was largely restored when activators were again added.

The results are in agreement with the view that ficin is active in a reduced form and inactive when oxidized by oxygen or other oxidants. The apparent coenzymic function of cyanide and sulfhydryl compounds actually consists in the protection of the enzyme against inactivation by oxidation or by combination with heavy metals.

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A COUPLING HISTOCHEMICAL AZO DYE TEST FOR ALKALINE PHOSPHATASE IN THE KIDNEY

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The relatively high concentration of alkaline phosphatase in the kidney suggests for this enzyme an important physiological rôle, the elucidation of which has been attempted by both chemical and histochemical methods. The latter methods are aimed at definitely establishing the loci of the enzyme activity in the kidney tissue. The basis of the currently used technique for histochemical demonstration is the formation of silver phosphate at the site of the alkaline phosphatase in the tissues. This technique involves successively hydrolysis of the glycerophosphate substrate, precipitation of the freed phosphate *in situ* as a calcium phosphate, conversion of the calcium salt by silver nitrate into silver phosphate with its subsequent blackening by sunlight. By means of this method or its modification, Gomori (1), Takamatsu (2), Hepler *et al.* (3), and Kabat and Firth (4) have shown this enzyme in large amounts about the brush border and within the lumen of the proximal convoluted tubule. The presence in the kidney tissue of inorganic phosphates, as well as those of the nucleoprotein and the phospholipids, detracts somewhat from the specificity of this technique. However, much of the inorganic phosphate and chloride as well may be removed from tissue sections by washing in distilled water before the test is performed. The question may be raised, however, as to whether the test medium is sufficiently alkaline to render soluble any silver chloride formed.

A new approach to the histochemical problem is the formation of an insoluble dye by utilization of the organic instead of the phosphate moiety of a phosphate ester, other than glycerophosphate, following splitting by the phosphatase. The essentials of this procedure are the hydrolysis of a monoaryl, instead of an alkyl organic phosphate, and immediately following the release of the aryl molecule its direct coupling with a diazotized amine to precipitate an insoluble dye at the site of the enzyme activity.

The formation of such an insoluble dye with the released β -naphthol of calcium β -naphthol phosphate is described in the following pages. The occurrence of the bulk of this dye in the proximal convoluted tubules of the kidney agrees generally with the renal distribution of alkaline phosphatase reported with other techniques.

Preliminary Procedures

The chemical details of the dye test were developed by one of us (M H G) The procedure required the selection of a suitable phosphate ester, as well as an acceptable diazotized amine, in order that the resultant azo dye compound would be rapidly formed, insoluble, and of a deep easily recognized color A suitable amine was readily available but, before a satisfactory enzyme substrate was obtained, several phosphate esters, mentioned below, were tested Phenyl phosphates were first tried Commercial disodium monophenyl phosphate was readily available for preliminary testing, but proved unsatisfactory because, although a dye was formed in the test-tube by reaction of the phosphatase-liberated phenol with any of the following diazotized amines, namely *p*-nitroaniline, toluidine, or α -naphthylamine, the dye formed in kidney sections was not sufficiently insoluble and was not retained *in situ* in the tissues Phenyl phosphates were accordingly eliminated

Members of the naphthol phosphate series were next tried Trial coupling reactions performed with β -naphthol and each of the following diazotized amines, namely *p*-nitroaniline, *p* aminoacetophenone, and α naphthylamine, yielded insoluble dyes of different colors and varying low solubility α -Naphthylamine was selected because of the deep red to purple color and extreme insolubility of the coupled product β Naphthol phosphate was not available commercially and it or its salt had to be prepared Barium β -naphthol phosphate, the first compound prepared, was insoluble in water An attempt was next made to prepare β naphthol phosphoric acid ester from the barium salt The two samples of the acid ester which were prepared were small in amount and contained traces of barium which apparently slowed the hydrolytic activity of the phosphatase in tissue sections Although paraffin sections of kidney, treated at a suitable pH with the acid ester prepared by sulfuric acid, showed the precipitated purple dye to be located at the expected site in the kidney tissue, the test was almost negative at the end of 2 hours and a period of 20 to 24 hours was needed for the dye to acquire its maximum intensity During this time secondary black dust-like deposits formed over the sections Calcium β -naphthol phosphate was the ester which finally gave satisfactory dye precipitates, ranging in color from a red to a deep red purple

Methods

Preparation of Reagents

Preparation of Monoaryl Phosphate Ester Barium β Naphthol Phosphate—Barium β -naphthol phosphate was prepared by a modification of King and Nicholson's method (5) for the preparation of phenylphosphoric

acid esters To 10 ml of phosphorus oxychloride, a solution of 15.8 gm of β -naphthol in 50 ml of dry pyridine was added with stirring The reaction was allowed to proceed for 20 minutes and the resulting β -naphthol phosphoryl chloride was decomposed with 5 ml of water A saturated aqueous solution of barium hydroxide was added until the mixture turned pink to phenolphthalein The barium β -naphthol phosphate precipitated almost completely and, after the mixture was chilled in the refrigerator, it was filtered with suction, washed with 50 per cent and then with absolute alcohol, and air-dried An attempt to convert the insoluble barium compound into the disodium β -naphthol phosphate failed because of the insolubility of the former

Preparation of β -Naphtholphosphoric Acid Ester—The barium β -naphthol phosphate was suspended in an equivalent amount of 4 N hydrochloric acid and the acid solution extracted with ether A small amount of acid ester crystallized out of the ether, but the ester was so difficultly soluble in ether that the yield was poor An unsuccessful attempt was then made to obtain the acid ester in greater yield by taking up the barium salt in dilute sulfuric acid, thereby precipitating barium sulfate and removing the precipitate by centrifugation This acid ester was also contaminated with traces of barium and furthermore much of the ester was hydrolyzed in preparation

Preparation of Calcium β -Naphthol Phosphate—Preparation of this ester was exactly like that described above for barium β -naphthol phosphate up to decomposition of the phosphoryl chloride At this point saturated aqueous calcium hydroxide was added until the reaction mixture became pink to phenolphthalein (approximately 18 gm in amount) The precipitate was filtered after being chilled and washed with 50 per cent alcohol, then with absolute alcohol, and air-dried The calcium salt of this ester was more soluble than the barium salt and gave a large yield An additional crop of calcium naphthol phosphate was obtained by adding alcohol to the combined filtrate and washings The calcium β -naphthol phosphate was readily hydrolyzed in the test-tube by the phosphatase contained in a thin slice of fresh kidney A strong coupling reaction obtained with diazotized naphthylamine yielded a red-purple dye in trial tests *in vitro* within as short a time as 10 minutes With the application of this chemical procedure to mounted sections of alcohol-fixed kidney the dye was visible to the naked eye in a section 6 μ in thickness in a few minutes

Diazotization of α -Naphthylamine

A slight excess, namely 2.5 instead of 2 equivalents, of 2 N hydrochloric acid was added to 0.2 gm (0.0014 mole) of α -naphthylamine suspended in

a little water. The use of freshly sublimed α -naphthylamine is advised in order to eliminate any degradation products of oxidation. Saunders ((6) p. 4) recommends the use of excess acid in order to insure complete diazotization. The mixture was shaken until the base was completely dissolved and the solution diluted to 50 ml. The solution was chilled to 10-12°, and to each 50 ml of amine 5 ml (1 equivalent) of a freshly prepared solution of sodium nitrite (0.9884 gm dissolved in 50 ml of water) were added. The diazotization solution was tested with starch iodide indicator and "balanced" by adding more nitrite or more naphthylamine hydrochloride as required until the end-point was reached (Saunders (6) p. 5). Diazotization was complete after the mixture had reacted for 15 minutes at 10°. The diazotized solution should be used immediately following completion of diazotization.

Preparation of Tissue

Young adult white rats of the Wistar strain and white mice, both with fasting for 24 hours and without, were killed by a blow on the head and the kidneys immediately removed and fixed. About 60 rats were used. Necropsy tissues from a few human kidneys were also studied. Slices of kidney 3 to 4 mm in thickness were fixed in 95 per cent alcohol or in cold acetone. Alcohol-fixed tissues were run through two changes of absolute alcohol to xylol. Acetone-fixed tissues were run through three changes of special pure acetone for 48 hours in the refrigerator and followed by benzene for 45 minutes. Both sets of tissues were then treated with two changes of paraffin, each of 2 hours duration in the thermostat, and embedded. Paraffin sections 6 μ in thickness, mounted on slides, were run through the xylol and alcohols to water just previous to immersion in the "dye" solution. Acetone fixation appeared to afford somewhat greater retention of phosphatase in the tissue, but was accompanied by a considerable amount of shrinking and distortion of the tissues owing to rapid dehydration.

Technique of Phosphatase Test

20 cc each of a substrate suspension of calcium β -naphthol phosphate (roughly 0.3 gm of dried ester in 250 cc of distilled water) and diazotized α -naphthylamine were mixed and the reaction immediately adjusted by a Beckman pH meter to the required pH by the addition of 6 N NaOH. Tests were always made at the optimal reaction of pH 9.4 and frequently at pH 9.0, 8.0, and 7.4 as well. Following adjustment of pH, 20 cc of buffer solution of the HCl-veronal buffer mixture of Michaelis (7) were added to obtain the desired pH. Upon the addition of the alkali a brown precipitate formed which was removed by rapid filtration through a Buch

nel funnel and quickly cooled to approximately 10° . The mounted tissue sections were immediately placed in a staining dish filled with this filtrate and slides were removed after incubation of 15 and 30 minutes and 1 and 2 hours in a refrigerator at 6° , washed in water, counterstained with light green if desired, and mounted in 50 per cent glycerol. The positive phosphatase reaction was manifested by the formation of a deep red to purple dye.

The intensity and speed of the reaction bear a direct relationship to the H ion concentration and to the temperature at which the enzymic hydrolysis is carried out. The optimal H ion concentration is at pH 9.0 to 9.4, where a maximum precipitate is formed. Definite though weaker reactions requiring a longer time to give a maximum precipitate occur at pH 8.0 and even at pH 7.4 with the kidney sections of most animals. The coupling of diazo compounds with the β -naphthol to form an insoluble azo dye occurs only in alkaline solution and this fact may be in part responsible for the less intense reactions observed at pH 7.4. Under the conditions of the experiment a slight fall in pH may occur during the course of the test. In the optimal 18 minutes incubation period this change in pH was not enough to affect the results noticeably and at the end of this period the concentration of active diazo compounds in the substrate mixture was still sufficient to give a heavy dark red precipitate with β -naphthol.

The "dye-coupling" test is characterized by an extreme rapidity of reaction. A strong deposition of the dye could be observed as early as 5 minutes after exposure of the phosphatase-containing tissues to the substrate preparations at the optimum pH. In 15 minutes at ice box temperature ($4-6^{\circ}$) a very intense reaction could be obtained. Further incubation only moderately increased the precipitate. Tests performed at 37° showed a correspondingly enhanced reaction, but incubation at $4-6^{\circ}$ was preferable in order to retard possible disintegration of the diazotized amine compound and formation of secondary products which are accelerated at higher temperatures upon prolonged incubation.

The dye which is precipitated in the tissues is not the actual phosphatase but a compound formed with the naphthol freed by the hydrolytic action of the enzyme. The dye reaction is complex and in addition to the specific red-purple precipitate, resulting from enzymic action in the tissue, secondary products may be formed during the diazotization which behave as simple diffusible stains. These stains diffuse through the tissue, giving a generalized yellowish background tissue color. This diffuse color does not alter the location or visibility of the phosphatase precipitate. To minimize or prevent such ancillary reactions, a high pH (9.0 to 9.3) should be established and the performance of the test not unduly prolonged.

Control Procedures

Certain preliminary control measures were observed in carrying out the test. Control tests performed upon the β -naphthol ester solution showed it to contain no free naphthol as determined by the color test for phenolic compounds with the Folin-Ciocalteu reagent. A further test carried out by exposure of this ester solution to serum known to contain phosphatase resulted in the freeing of sufficient β -naphthol by hydrolysis of the phosphate ester to produce with the Folin-Ciocalteu reagent the appearance of a deep blue color which was absent in phosphatase-free serum controls. It was demonstrated, therefore, that the ester preparation employed contained no free β -naphthol but could be hydrolyzed by substances containing phosphatase with the resultant liberation of β -naphthol.

A second series of control tests, performed upon the diazotized α naphthylamine solutions used, showed that the addition of the β -naphthol ester to properly buffered solutions produced no red dye precipitates in the absence of enzymic substances. If, however, a small quantity of β naphthol was added to the alkaline diazo solution, an immediate red dye precipitate was formed.

Control histochemical tests in which prepared tissue sections were incubated with alkaline buffered diazo compounds, in the manner of the regular procedure but with the omission of β -naphthol phosphate substrate, exhibited no red dye precipitate.

Control reactions gave considerable evidence of the inhibiting effect of heat. Immersion of rat tissue sections, immediately before testing for phosphatase, in a water bath at a temperature of between 75–80° for 10 minutes resulted in complete inactivation of the phosphatase, as shown by absence of any color reaction.

In view of the general instability of all diazo compounds it is necessary to carry out the diazotization of the α -naphthylamine immediately prior to use in the test and to allow as little time as possible to elapse between completion of diazotization, mixing the ester substrate, pH adjustment and addition of buffer, and the immersion of the tissues for testing. Even under apparently optimal conditions with a short incubation period (15 to 18 minutes) at 4–6°, yellow-brown disintegration products may form in the solution. It is possible that the test may be further refined by procedures aimed at stabilizing the diazo compound. In its present form the test is intricate and probably is not adapted to routine use without modification.

Results

The histochemical dye technique for alkaline phosphatase has been extensively used on tissue from mouse, rat, and human kidneys. It may be briefly stated here that our results in general confirm those reported with

the Gomori and Takamatsu techniques. The phosphatase occurred mainly in the cortex, where it was observed in definite locations. The medulla was for the most part free of the enzyme. Species variations and minor differences due to physiologic conditions or pathologic changes will be published elsewhere. Our results premise that the principle of azo dye formation by means of coupling with products mediated by tissue phosphatase may be applicable to the histochemical demonstrations of other enzymes in tissues.

SUMMARY

1. A histochemical test for alkaline phosphatase based on the precipitation of an insoluble azo dye formed by coupling of β -naphthol, derived from hydrolysis of calcium β -naphthol, and diazotized α -naphthylamine has been described.

2. With the dye test, the location of the phosphatase in the kidney agrees substantially with that obtained by the silver technique of other investigators.

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THE COLORIMETRIC ESTIMATION OF PROTEIN-BOUND SERUM IODINE*

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The following procedure is an adaptation of the precipitation and digestion procedures described by Man, Smirnow, Gildea, and Peters (1) and Riggs and Man (2) to distillation in the Chaney apparatus (3) as modified by Riggs¹ and application of photoelectric-micro colorimetry to the quantitative assay of iodate instead of the usual starch-iodine titration. This procedure permits satisfactory analyses to be made on 4 cc of serum.

Reagents—The reagents for precipitating the serum proteins were prepared according to the directions of Man, Smirnow, Gildea, and Peters (1). All other reagents were prepared according to the directions of Riggs and Man (2) as outlined by Salter (4).

"Water pump" dry nitrogen (Air-Reduction Sales Company)

The solutions of arrowroot starch and of sodium bisulfite (NaHSO_3 , Merck, C P) were prepared freshly at monthly intervals.

Special Apparatus—Riggs' modification of the distillation apparatus originally described by Chaney (3) (see Fig 1). The distillation flask of this apparatus is used for the digestion of the sulfuric acid solution of the serum proteins. The central section of the apparatus (i.e. that portion lying between Joints A and B) should be washed with chromic acid cleaning solution followed by several rinsings of distilled water between runs. No stop-cock grease is used on the glass joints, but a trace may be used on the stop-cock attached to the "trap" and on the stop-cock of the dropping funnel. Care should be taken to prevent any contamination of the distillation mixture with the lubricant.

Evelyn photoelectric micro colorimeter, equipped with 2 cc cells and a filter having maximum light transmission at 620 $m\mu$ (range 525 to 660 $m\mu$).

All apparatus used must be scrupulously clean.

Procedure

Collection of Blood—An appropriate quantity (usually 8 to 10 cc) of venous blood is drawn, care being taken to avoid areas on the skin which may have been contaminated with iodine.

* This work was supported by a grant from the Commonwealth Fund of New York.

¹ Riggs, D S, personal communication.

Separation of Protein-Bound Serum Iodine—This is carried out essentially according to the directions of Man *et al* (1). Briefly, 2 to 4 cc of serum are precipitated by the addition of 32 cc of a solution of zinc sulfate in sulfuric acid followed by 4 cc of sodium hydroxide solution. The protein precipitate which forms is collected upon a 9 cm sintered glass filter of coarse grade. It is repeatedly washed on the filter with 20 to 30 cc portions of distilled water until the filtrate gives no precipitate (AgCl) with acidified 0.1 N silver nitrate solution.

The washed precipitate, which is considered to be free from inorganic iodine, is transferred quantitatively to the digestion distillation flask with the aid of 80 cc of 18 N sulfuric acid, in which the precipitate is soluble.

Digestion—Two small pieces of copper metal, approximately 10 mg of ceric sulfate, and 5 gm of potassium permanganate are added to the 18 N sulfuric acid solution of the protein. An approximately 10 to 12 inch thermometer (0–200° calibration) is placed in the side neck of the digestion flask. Two glass beads are added as antibumps. The flask is placed under a hood and heated with a Bunsen burner. To avoid foaming, heating should be slow at first, later the flame is increased and the digestion is continued until the temperature reaches 195–200°. The flame is then extinguished. When the digestion mixture has cooled to 100°, about 22 cc of distilled water are added,² some being used to rinse down the necks of the flask and the thermometer which is removed at this time. After the water has been added, the flask must be agitated gently for a moment to mix the contents.

Distillation of Iodine—The chemically clean distillation apparatus is prepared for use by introducing 1 cc of 0.1 N sodium bisulfite solution and 1 cc of 1 M potassium carbonate solution into the trap (see Fig 1) with the aid of pipettes introduced through the upper opening (Joint A) of this section of the apparatus. The condenser is then attached as shown in the figure.

After a 5 inch thermometer (100–200° calibration) has been placed inside the digestion flask, it is attached to the lower end of the distillation apparatus (Joint B). Thus the digestion flask becomes a distillation flask. A dropping funnel containing saturated oxalic acid solution ($\pm 21^\circ$) is placed in the side neck of the distillation flask as shown in Fig 1. The mixture in the flask is heated with a Bunsen burner to a temperature of 138–143°. During the heating period, the apparatus is occasionally tilted slightly to mix the contents of the distillation flask.³ While the tempera-

² The volume of water added is of importance with relation to the maintenance of a suitable distillation temperature (see the next section). If too little water is added, it is difficult to prevent overheating and *vice versa*.

³ In tilting the apparatus, one must take care not to cause the contents of the trap to spill or be sucked back into the distillation flask.

ture is maintained within these limits, the oxalic acid solution is added drop by drop to the distillation flask. After the mixture in the distillation

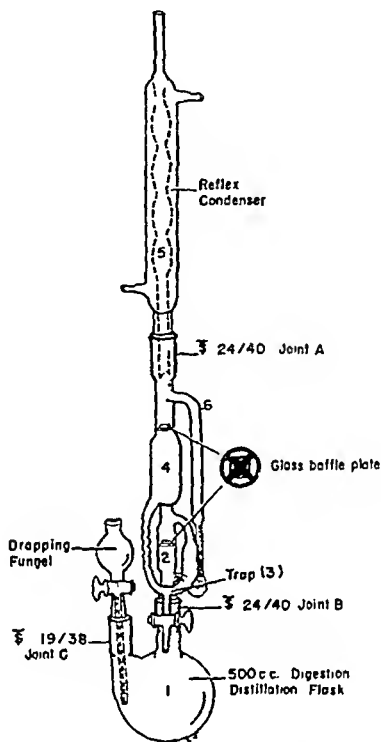


FIG 1 Riggs distillation apparatus. The 2-necked flask (1) is used both for digestion and for the distillation of iodine. During the distillation procedure, the digested solution in flask (1) is heated. Steam is generated which passes up column (2), past the glass baffle plate, to the right, and downwards through the trap (3). The steam then courses to the left and upwards to chamber (4). From there it passes by the second baffle plate and into the condenser (5). The water of condensation drops down and is returned by tube (6) to column (2), whence it drops back into the distillation flask (1). Thus the apparatus permits reflux distillation of the iodine into the trap (3). The dropping funnel inserted into the side neck of flask (1) is used for introducing the oxalic acid. The total length of the apparatus is 85 cm. It was made by the Macalaster-Bicknell Company, New Haven, Connecticut.

flask becomes colorless, 30 drops more are added and the distillation is continued thereafter for 5 minutes. By that time, the iodine will have been completely distilled to the solution in the trap, where it is reduced to potassium iodide.

When the distillation is completed, the flame is extinguished and the contents of the trap are drained through the adjacent stop-cock into a small Erlenmeyer flask. To insure quantitative drainage, the trap is rinsed three times with 1 cc portions of distilled water added through the opening of Joint A. The combined distillate and water rinsings collected in the Erlenmeyer flask are slowly evaporated to a volume of approximately 1 to 2 cc upon an electric hot-plate. The contents of the flask are then transferred quantitatively with minimum distilled water rinsings to a 10 cc round bottom Pyrex test-tube approximately 115 mm long and 12 mm in internal diameter, with a slight constriction about 1 cm from the top and graduated at 1.0 cc.⁴

Groak Reaction—The purpose of this reaction is to oxidize the iodide in solution in the test-tube to iodate for colorimetric assay. The test-tube is placed in the water bath, the temperature of which is maintained between 70–80°. After 2 minutes, the tube is removed for the addition, with shaking, of 6 drops (0.5 cc)⁵ of 1 N potassium permanganate solution. *Care should be taken so to deliver this solution that little or none falls on the wall of the tube above the constriction.* The tube is replaced in the water bath and at the end of 4 minutes is withdrawn for the addition, with shaking, of 10 drops (0.6 cc) of 8 N sulfuric acid solution. The acid solution should be delivered near the mouth of the tube so that it tends to wash down the walls of the tube. After the tube has been heated in the water bath for another period of 4 minutes, it is withdrawn and 0.75 N sodium nitrite is added drop by drop with shaking until the solution becomes crystal-clear and colorless. The tube should then be tilted and rotated so that the walls of the tube are thoroughly washed with the contents. Immediately thereafter, 2 drops of 5 M urea solution are added. The tube is again shaken briefly and then tilted and rotated *so that the contents wash the walls of the tube up to the lip.* The addition of nitrite solution and urea should not consume more than 1½ minutes.⁶

The tube is then replaced in the water bath (70–80°) in order to evaporate the solution down to a volume of 1.0 cc. A stream of clean dry nitrogen instead of contaminated laboratory air is blown over the solution in the tube to hasten the evaporation. The tube should be shaken occasionally *to prevent the losses of iodate which occur if the solutes dry out on the inner walls of the tube.*

⁴ Although the constriction is not essential, it helps prevent spilling during the Groak reaction.

⁵ The values in parentheses give the volume equivalent of the drops delivered.

⁶ There are potential but easily avoidable difficulties inherent in the Groak reaction. Both permanganate and nitrous acid (formed from sodium nitrite) give an intense blue color with starch in the presence of iodide. Hence it is important to eliminate completely the permanganate with nitrous acid and the nitrous acid with urea by careful mixing of solutions and washing of the walls of the test tube.

Colorimetric Assay—Under the conditions used here iodate in the presence of iodide, sulfuric acid, and starch reacts to give a blue color, the intensity of which is directly proportional to the amount of iodate. The tube containing exactly 1 cc of solution is thoroughly chilled in a beaker of ice water (0–5°). 0.1 cc of a 1 per cent starch solution is added and the tube is shaken briefly to mix. 0.06 cc of a 0.2 per cent potassium iodide solution is then added. The tube is shaken briefly and returned to the ice water bath pending colorimetric assay. A blank is prepared simultaneously as follows. To 0.3 cc of 8 N sulfuric acid solution in another 10 cc test-tube, distilled water is added to make the volume up to 1.0 cc. The mixture is chilled and 0.1 cc of starch solution and 0.06 cc of iodide solution are added as described above.

The color formed is stable at ice water temperature for at least 10 minutes, but may fade if allowed to stand for longer periods of time. Approximately half a minute prior to colorimetric assay, the tube containing the solution to be read is transferred to a beaker containing water at 15°. 1.0 cc of the solution are then pipetted into a 2 cc micro colorimeter cell.⁷ With a filter having maximum transmission at 620 m μ (range 525 to 660 m μ) the absorption cell containing the blank solution is set in place in the micro colorimeter and the galvanometer is adjusted to 100. This cell is then replaced by that containing the unknown sample, and the galvanometer reading is recorded. The quantity of iodate present is calculated from this galvanometer reading by referring to a calibration curve in which galvanometer readings are plotted against known amounts of iodine as potassium iodate (0.1 to 0.6 γ) treated according to the directions outlined above for the blank. Or, when the galvanometer reading of the blank is set at 100,⁸ the micrograms of iodine in the sample analyzed may be calculated from the equation $C = 1/K (2 - \log G)$, when K is the constant of proportionality determined from solutions containing known amounts of iodine (see Table I) and G is the galvanometer reading of the unknown.

EXPERIMENTAL

Extinction-wave length curves of the blue-colored compound formed by potassium iodate and starch in the presence of potassium iodide and sulfuric acid were obtained with the aid of light filters. These showed that the maximum light extinction of the colored solution was at approximately 620 m μ . Essentially identical curves were obtained with iodate prepared from serum proteins by the analytical procedure outlined above.

⁷ An Evelyn micro colorimeter with an open type absorption cell and a Rubicon Filter 620 is used in this laboratory.

⁸ When the galvanometer has been adjusted to 100 with a cell containing 1.0 cc of water in place, the blank described above should give a galvanometer reading between 98 and 100.

The data obtained from sample experiments in establishing our constant of proportionality (K in the equation above) are presented in Table I. They indicate that pure iodine, as potassium iodate, in amounts ranging from 0.132 to 0.529 γ per sample analyzed may be determined with an error of approximately 5 per cent. The suggested linear proportionality between the color developed and the iodate concentration has been amply confirmed by other similar analyses.

To determine the recovery of iodine at various steps in the analytical procedure, known amounts of potassium iodate in distilled water were subjected to (a) the Groák procedure, and (b) the digestion, distillation, and Groák procedures. The iodine recovered as iodate was determined colorimetrically.

TABLE I

Three Experiments Taken from Series Establishing Proportionality Constant (K) between Concentration (C) of Iodine and Galvanometer Reading (G) in Equation $C = 1/K (2 - \log G)$

In these experiments the galvanometer was adjusted to 100 with the blank in place

Iodine as iodate	Galvanometer reading			K		
	Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
γ						
0.132	80.5	81.0	80.0	0.713	0.693	0.734
0.265	65.0	65.0	63.5	0.706	0.706	0.745
0.397	52.5	52.0	52.0	0.705	0.715	0.715
0.529	41.5	41.0	41.0	0.722	0.731	0.731
Average				0.712	0.712	0.731

Table II indicates that the average recovery in the Groák procedure alone is 99 per cent (range 91 to 107 per cent) and in the complete procedure 98 per cent (range 91 to 103 per cent). Blanks containing no added iodine gave satisfactorily low values.

Table III gives data concerning the recovery of iodate added to an acid solution of washed serum protein. In these experiments 8 cc of serum were precipitated according to the analytical procedure. After the precipitate had been washed with distilled water, it was dissolved in 160 cc of 18 N sulfuric acid. This solution was divided into two 80 cc portions. To one portion nothing was added, to the other 0.529 γ of iodine was added. Both portions were then assayed for their iodine content according to the analytical procedure. The results obtained show that the average recovery of added iodine was 94 per cent of the theoretical (range 87 to 103 per cent).

Table IV reports duplicate determinations of the concentrations of pro-

ten-bound serum iodine on ten essentially normal individuals 2 cc of serum were used for one of the duplicates, 4 cc for the other It is seen

TABLE II

Recovery of Iodine Added to Water before Groök Procedure and before Combined Digestion, Distillation, and Groök Procedures

	Experiment No	Theoretical (1)	Determined (2)	Ratio $\frac{(2)}{(1)}$	Average
		γ	γ		
Groök procedure alone	1	0	0 01		
	2	0 132	0 143	1 07	
	3	0 132	0 120	0 91	
	4	0 132	0 126	0 96	
	5	0 264	0 264	1 00	
	6	0 529	0 515	0 98	
	7	0 529	0 529	1 00	0 99
Combined digestion, distillation, and Groök procedures	8	0	0 005		
	9	0	0 000		
	10	0 132	0 120	0 91	
	11	0 132	0 136	1 03	
	12	0 264	0 254	0 96	
	13	0 529	0 529	1 00	
	14	0 529	0 515	0 98	0 98

TABLE III

Recovery of Iodine Added to Acid Solutions of Serum Protein

Experiment No	Iodine added	Iodine recovered		Ratio $\frac{(1)}{(2)}$	Average
		Determined (1)	Theoretical (2)		
	γ	γ	γ		
1a	0	0 192			
1b	0 529	0 624	0 721	0 87	
2a	0	0 174			
2b	0 529	0 725	0 703	1 03	
3a	0	0 267			
3b	0 529	0 708	0 796	0 89	
4a	0	0 255			
4b	0 529	0 742	0 784	0 95	
5a	0	0 186			
5b	0 529	0 731	0 715	1 02	
6a	0	0 186			
6b	0 529	0 638	0 715	0 89	0 94

that none of the duplicate values differed from the other by more than 1 0 γ per cent (Sample 7) and that in most instances the difference was 0 3 γ per cent or less

The clinical application of the method is indicated by the data presented in Table V, giving the protein-bound serum iodine concentrations of 50 normal individuals of various ages and of eight patients with either hypo- or hyperthyroidism. It is noted that the average value for normal children (5.5 γ per cent) was lower than the average value for normal adults (7.0 γ per cent). The values for patients with hypothyroidism were below the

TABLE IV

Duplicate Analyses of Protein Bound Serum Iodine Concentration, Expressed As Micrograms Per Cent

Sample No	Volume of serum analyzed		Difference (1) - (2)
	2 cc (1)	4 cc (2)	
1	6.1	5.8	0.3
2	7.2	7.3	-0.1
3	6.5	6.6	-0.1
4	6.7	6.7	0.0
5	7.6	7.7	-0.1
6	6.3	6.5	-0.2
7	8.5	7.5	1.0
8	8.4	9.0	-0.6
9	9.9	9.8	0.1
10	4.4	4.7	-0.3

TABLE V

Protein Bound Serum Iodine Concentrations of Normal Children and Adults and Hyperthyroid and Hypothyroid Subjects

Condition	No of subjects	No of determinations	Protein bound serum iodine	
			Average	Range
			γ per cent	γ per cent
1 Normal children (3-13 yrs.)	39	39	5.5	4.0-7.0
2 " young adults	11	17	7.0	6.0-8.4
3 Juvenile hypothyroidism or cretinism	4	8	2.3	1.8-3.0
4 Myxedema in adult	1	1	3.8	
5 Thyrotoxicosis in adults	3	4	12.6	9.2-14.5

lower limit of the normal values, while those for patients with hyperthyroidism were above the upper limit of normal.

Comments

Because it is well known that a number of factors influence the quality and intensity of the color formed by starch and iodine (2), a study was made

of the effect of moderate variations in experimental conditions upon the accuracy of the colorimetric assay described here

Although the quality of sulfuric acid is of importance (2), with pure iodate solutions the quantity present at the time of color formation may be doubled or halved without altering the color formation. Likewise, while the color formed is the same whether 0.03 or 0.09 cc. of arrowroot starch solution is used, the colors formed with other brands of starch were much weaker. The addition of between 1 and 3 drops of 5 M urea solution is also without effect upon the quality or intensity of the blue starch-iodine color. However, large quantities of urea do inhibit the color formation slightly.

On the other hand, certain other factors have an important influence upon the color assay. The sensitivity of the color reaction is diminished when the concentration of iodate iodine is much less than 0.1 γ per cc. This means that in the analysis of a 4 cc. sample of serum, when a 1 cc. volume is used for the colorimetric analysis, the concentration of protein-bound serum iodine can be determined with accuracy only for values of 2.5 γ or more per 100 cc. of serum. Thus any increase in the volume of the solution assayed colorimetrically would require a corresponding increase in the volume of serum analyzed to maintain sensitivity.

Another factor of importance is the temperature of the iodate solution to be assayed colorimetrically. There is a definite tendency for the starch-iodine color to be less intense and to fade at temperatures above 17°. However, the color developed is essentially the same at temperatures between -4° and +15°, and no fading occurs during a 10 minute period at 0-4°. These observations are in general agreement with those of other workers (2, 5).

Riggs and Man have observed that in the presence of the large amount of salts in the solution after the Groök reaction an excess of potassium iodide results in a reddish violet starch-iodine color which is difficult to titrate with thiosulfate (2). On the other hand, if the quantity of iodide added is insufficient, the iodide-iodate reaction does not go to completion and titration values are low. They concluded that for amounts of iodate up to 2 γ , 0.06 cc. of 0.2 per cent potassium iodide is satisfactory. Similar observations have been made in this laboratory with the present colorimetric method. The values obtained with solutions of iodate developed according to the procedure outlined above for the blank are satisfactory provided 0.06 cc. of a 0.2 or 0.4 per cent potassium iodide solution is added. With solutions of iodate prepared with the aid of the Groök reaction, satisfactory values are obtained when 0.06 cc. of a 0.04 to 0.4 per cent iodide solution is used. The use of larger quantities of iodide than those described above yields variable colors and irregular assay values. When smaller quantities are used, the colors formed are of satisfactory quality, but their assay values are low.

SUMMARY

The Riggs and Man procedure for the determination of the protein bound serum iodine has been shortened and modified to permit the colorimetric determination of the iodine

The determination may be carried out with an accuracy of ± 10 per cent on a single 4 cc sample of serum

Preliminary observations suggest that the procedure provides a convenient and accurate estimation of thyroid function

We are indebted to Dr D S Riggs and Dr E B Man for their generous advice during the course of this work

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CHOLINE AND THE PREVENTION OF HEMORRHAGIC KIDNEYS IN THE RAT

II PHOSPHOLIPID TURNOVER DETERMINED WITH RADIOACTIVE PHOSPHORUS

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The production of kidney lesions in rats fed a diet deficient in choline has been clearly established by Griffith and his associates (1). A previous report from this laboratory (2) showed that the percentage concentration of phospholipid in the kidneys and livers of young rats, maintained for 10 days on a choline-deficient diet, was lower than that found in similar rats which had been supplied with choline. It was suggested that a possible explanation for the development of renal damage was a failure in phospholipid formation at a period when phospholipids were needed for the development of the growing kidney. This explanation has been further investigated with the aid of radioactive phosphorus (P^{32}). While Perlman and Chaikoff (3) have used this technique to study the effects of choline upon phospholipid turnover, their results were not secured under the special circumstances needed for the production of kidney lesions.

The age, weight, sex, diet, and vitamin supplements of the rats were the same as previously reported (2). Three series of animals were used. Series A and B were each composed of two groups of ten animals. One group in each series received 3 mg of choline per gm of the basal diet, the other group of rats was maintained without choline. On the 9th experimental day, the animals were fasted for 12 hours, after which each animal received by injection 1 cc of an isotonic solution of disodium hydrogen phosphate containing radioactive phosphorus. 5 hours after the injection, the animals were killed by stunning. The livers and kidneys were removed and pooled for phospholipid estimation according to the oxidation method of Bloor (4). Measurements of the radioactivity of the phospholipid fraction were made from thin deposits on Petri dishes with a thin walled β -ray counting tube and an improved counting rate meter. Series C contained thirty-five rats, 21 days old at the beginning of the experiment. These animals received the basal diet plus 3 mg of choline per gm of food. Phospholipid concentration and turnover were measured in the livers and kidneys of these rats at frequent intervals during a period of 30 days.

Results

Table I records the average phospholipid concentration and average percentage of the administered radioactive phosphorus recovered per gm of phospholipid and per 100 gm of tissue for both the livers and kidneys of Series A and B. Fig 1 shows the average percentage concentration of phospholipid in the kidney of the choline-fed rat over the 30 day experimental period in Series C and also the average percentage of the administered radioactive phosphorus recovered (a) per gm of liver and kidney phospholipid and (b) per 100 gm of liver and kidney tissue.

The addition of choline to the basal diet markedly stimulated phospholipid turnover in both the liver and kidneys as measured by the percentage of the administered radioactive phosphorus recovered from these organs. Choline maintained the percentage concentration of phospholipid in the

TABLE I

Phospholipid Concentration and Turnover in Young Rats on 10th Experimental Day

Series	Group No	Treatment	Concentration of phospholipid		Per cent recovery of radioactive phosphorus			
			Kidney	Liver	Per gm phospholipid		Per 100 gm tissue	
					Kidney	Liver	Kidney	Liver
A	1	No choline	1353	2135	6.1	8.3	8.3	17.5
	2	Choline	2597	2472	8.0	12.5	20.6	31.4
B	3	No choline	1563	1880	6.2	10.5	9.7	20.0
	4	Choline	2500	2450	7.4	14.0	18.2	30.0

liver and kidneys at a high level. All animals not receiving choline showed fatty livers and renal damage, the percentage concentration of phospholipid in the kidneys was reduced to a very low level. In Series A, 24 per cent more radioactive phosphorus was recovered per gm of phospholipid from the kidneys of rats receiving choline than from those not receiving choline, in Series B the difference was 16 per cent greater recovery in favor of the choline-treated animals. The ratio of the per cent administered radioactive phosphorus recovered per gm of kidney phospholipid from choline-treated animals to that in non-choline-treated animals, for Series A and B, was 1.3 and 1.2 respectively.

Similar results were obtained in the case of liver phospholipids. In Series A 34 per cent and in Series B 25 per cent more radioactive phosphorus were recovered per gm of phospholipid from the livers of the choline treated animals than from the livers of the animals which did not receive

choline. The ratios were 1.5 and 1.3 for the livers of Series A and B respectively.

If the comparison of the percentage recovery of administered radioactive phosphorus is made on the basis of 100 gm. of tissue, the kidneys of the choline-treated animals were 60 per cent more active in Series A and 47 per cent more active in Series B than the kidneys of the untreated animals, the respective ratios were 2.5 and 1.9. Expressed on this basis,

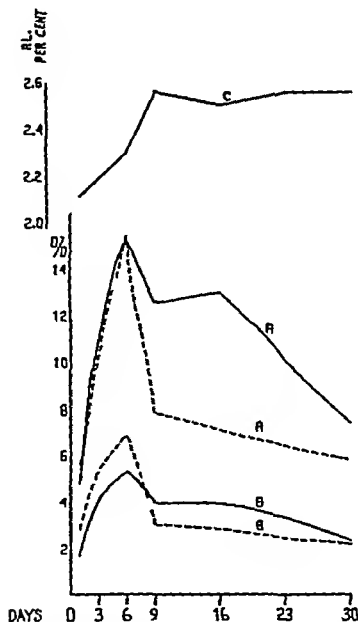


FIG. 1. Phospholipid turnover and concentration in young rats receiving choline. The solid line denotes liver, the dash line, kidney. Curve A, per cent recovery of radioactive phosphorus per 100 gm. of tissue; Curve B, per cent recovery of radioactive phosphorus per gm. of phospholipid; Curve C, phospholipid concentration in kidney.

the livers of the choline-treated animals were 44 per cent more active in Series A and 33 per cent more active in Series B than the livers of the choline-deficient animals, the ratios being 1.8 and 1.5.

The results of Series C show that the percentage concentration of phospholipid in the kidney increases during the first 9 days on the experimental diet, after which time the concentration is maintained at a constant level. The phospholipid turnover in both the liver and kidneys is smallest on the

1st experimental day, increases to a maximum on the 6th day, and then declines to the 30th day on the experimental régime

DISCUSSION

Griffith (1) reported that renal lesions and fatty livers were most readily produced, on a choline-deficient diet, within 7 to 10 days in 40 gm male rats 20 to 30 days old. The kidney lesions developed over a 24 to 48 hour period, between the 6th and 9th days. A marked decrease in the incidence of renal degeneration was found in older and heavier rats. The renal degeneration in our animals invariably occurred between the 8th and 10th days.

The experimental data of a previous study (2) showed that renal damage is preceded by a marked fall in the percentage concentration of phospholipid in the kidneys. Choline increases the concentration of phospholipid in the kidneys and stimulates phospholipid turnover. A similar stimulating effect of choline on phospholipid turnover in the liver of the fat-fed rat has been reported by Perlman and Chaikoff (3). The results of Series C indicate that the highest turnover of phospholipid and, therefore, the highest requirement for choline to form phospholipid is on the 6th experimental day. If choline is absent from the diet at this crucial time, the phospholipid turnover is not sufficiently rapid to meet the demand for phospholipid formation. The phospholipid turnover is markedly reduced in older rats. This explains why the choline requirement is significantly less in rats over 35 days of age and why rats of this age are more resistant to choline deficiency than younger animals. The decreased requirement for choline might explain the spontaneous recovery noted by Griffith (1) in animals which survived the initial 10 day period.

SUMMARY

The results of a previous study, that choline deficiency causes a diminished concentration of phospholipid in the livers and kidneys of young rats in which kidney damage is produced by the dietary régime, have been confirmed. Measurements of recovery of radioactive phosphorus from tissue phospholipids show that the choline deficiency decreases phospholipid turnover. In normal young rats the phospholipid concentration and phospholipid turnover in the kidneys are greatest in the brief period in which choline deficiency causes marked renal lesions. These results support the hypothesis that the kidney damage is consequent to an inadequate supply of phospholipid.

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A STUDY OF THE ACETYLATION IN VIVO OF CERTAIN *d*-AMINO ACIDS*

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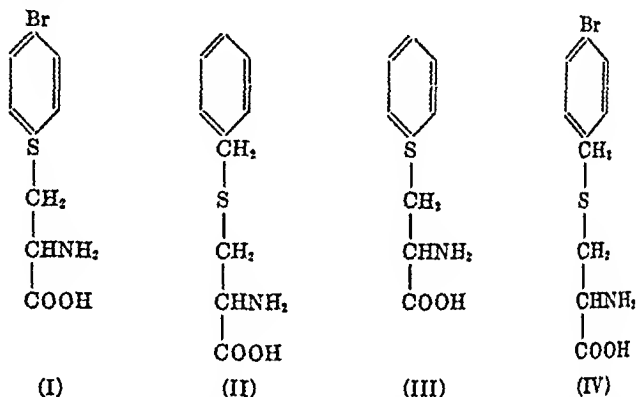
During studies on the inversion of *d*-amino acids to their antipodes within the animal body it had been established that *d*(-)-phenylaminobutyric acid can be converted to and excreted as the acetyl derivative of *l*(+)-phenylaminobutyric acid (1). It then became of interest to determine whether other amino acids which are excreted as the *N*-acetyl derivatives likewise undergo optical inversion of the configuration of the α -amino carbon atom. The hypothesis was tested by feeding *S*-benzyl-*d*-homocysteine and *S*-benzyl-*d*-cysteine (2). Both of these were found to be inverted. The isolation of a small amount of the *dl*-acetyl derivative of benzylcysteine (2) suggested the possibility that direct acetylation may occur without optical inversion of the *d*-amino acid (3). In a later investigation (4) *p*-bromophenyl-*d*-cysteine was fed and the *N*-acetyl-*p*-bromophenyl-*d*-cysteine isolated from the urine had the sign of optical rotation characteristic of the *d* enantiomorph. Direct acetylation was thus established unequivocally, although the comparison of the numerical value of the isolated derivative with that of the optically pure acetyl derivative prepared *in vitro* showed that to some extent inversion had also taken place.

In the experiments on benzyl-*d*-cysteine reported earlier (2) we had repeatedly crystallized the excreted acetyl derivative to obtain a product for characterization. By so doing it was possible that some *l* isomer had been separated from the accompanying racemic isomer. The feeding of benzyl-*d*-cysteine has been repeated and fractional crystallization was therefore avoided in the isolation of the acetylated product in order that more significance could be attached to the numerical value of the rotation as well as to the direction of rotation. When this was done, the lower rotation of the acetylbenzylcysteine isolated indicated somewhat larger amounts of *d* isomer than had been previously detected, although inversion still predominated. The data of the benzyl-*d*-cysteine feeding and the subsequent experiments with other compounds are given in Table I. The contrast between the behavior of the benzyl-*d*-cysteine *in vivo* with that of

* The experimental work reported in this paper was carried out during 1939-40. The presentation of the work has been unavoidably delayed.

p-bromophenyl-*d*-cysteine which for the most part underwent direct acetylation still remained

The structures of *p*-bromophenylcysteine (I) and benzylcysteine (II) are seen to differ only in the nature of the group attached to the sulfur of the cysteine moiety. We were led, therefore, to investigate whether the property of *p*-bromophenyl-*d*-cysteine which promoted the considerable amount of direct acetylation was the presence of a bromine atom attached to the benzene ring or the direct attachment of an aromatic nucleus to the sulfur. It seemed possible to differentiate between these factors by studying phenyl-*d*-cysteine (III) and *p*-bromobenzyl-*d*-cysteine (IV). In the



case of the *p*-bromobenzyl-*d*-cysteine, the rotation of the acetyl derivative isolated from the urine indicated a preponderance of the *d* isomer. The *p*-bromobenzyl-*d*-cysteine thus was similar in behavior to the *p* bromophenyl analogue, that is, direct acetylation predominated. On the other hand, after the feeding of phenyl-*d*-cysteine, the isolated acetylphenyl cysteine consisted mainly of the *l* isomer. In other words, inversion predominated in the acetylation process. In this respect phenyl-*d*-cysteine more closely resembled benzyl-*d*-cysteine.

From the foregoing results, the presence of a bromine in the aromatic nucleus rather than the direct attachment of the aromatic nucleus to the cysteine appeared to be the more important factor contributing to the tendency of the animal body to bring about direct acetylation of amino acid derivatives of this type. We cannot, however, assign the promotion of direct acetylation exclusively to the presence of bromine attached to the aromatic nucleus. Further studies revealed that the feeding of *o* bromobenzyl-*d*-cysteine led to the excretion of an optically inactive acetyl derivative, a finding which indicates equal amounts of direct acetylation and inversion. On the other hand, we found that *p* bromobenzyl-*d* homo-

cysteine, like benzyl-*d*-homocysteine, showed little or no direct acetylation, since the excreted compound was almost entirely the *l*-amino acid derivative

In the course of this investigation, we have obtained further evidence that the process of inversion is not the result of racemization of the acetyl derivative. The derivatives of *l*-cysteine corresponding to those already discussed were fed and in confirmation of previous reports (1-7) the excreted acetylated products were optically pure derivatives of the *l* isomers. In no case have we found any evidence for the inversion of the *l*-amino acid configuration in a feeding experiment. Similarly, in no case within our experience have we found an occurrence of the inversion of an acetyl derivative of a *d*-amino acid configuration. Nevertheless, as a further check, *N*-acetylbenzyl-*d*-cysteine was fed to rats. The excreted acetyl derivative isolated from the urine showed no change from the fed material in the sign or magnitude of the optical rotation. Thus it can be categorically stated that, when inversion occurs, it is not the result of the racemization of the acetyl derivative.

In addition it should be noted that in the formation of mercapturic acids following the ingestion of bromobenzene (4), benzyl chloride (5), and *p*-bromobenzyl bromide (6) there is no indication of the presence of any of the *d* isomer.

EXPERIMENTAL

Preparation of Cysteine Derivatives

o-Bromobenzyl-*d*-cysteine—12.9 gm of benzyl-*d*-cysteine were reduced by metallic sodium in liquid ammonia. 13.0 gm of *o*-bromobenzyl chloride dissolved in 50 cc of absolute ether were added to the solution and stirring was continued for about 1 hour. The derivative was isolated and purified in the usual manner (2). 4.8 gm of the derivative decomposing at 203-205° were obtained. It possessed a rotation of $[\alpha]_D^{21} = -19^\circ$ for a 1 per cent solution in 1 *N* NaOH. The nitrogen content of 4.89 per cent agreed closely with the theoretical value of 4.82 per cent.

p-Bromobenzyl-*d*-cysteine—This compound was first made by treating the reduction product of benzyl-*d*-cysteine and sodium in liquid ammonia solution with *p*-bromobenzyl chloride in the same manner described for *o*-bromobenzyl-*d*-cysteine. It was later found more convenient to add the appropriate halide to sodium *dl*-cysteinates in liquid ammonia and then isolate the *p*-bromobenzyl-*dl*-cysteine (m.p. 214-216°) and resolve it in the manner described for benzyl-*dl*-cysteine (8). The *dl*-formyl derivative crystallized from acetone-benzene mixtures melted at 148°. The brucine salt of the *N*-formyl-*p*-bromobenzyl-*d*-cysteine was crystallized from

butanol to a constant rotation of $[\alpha]_D^{13} = -21.0^\circ$ for a 1 per cent solution in water. *p*-Bromobenzyl-*d*-cysteine was obtained with a decomposition point of $211-213^\circ$ and a rotation of $[\alpha]_D^{15} = -28.0^\circ$ for a 1 per cent solution in 1 *N* NaOH. The nitrogen content of 4.74 per cent agreed closely with the theoretical value of 4.82 per cent.

p-Bromobenzyl-*l*-cysteine—The filtrate from the first precipitation of the brucine salt of formylbromobenzyl-*d*-cysteine described above was concentrated to 0.5 volume and allowed to stand several days in the ice box. A small amount of precipitate was removed by filtration and the filtrate was concentrated to a thick syrup. This syrup was suspended in 1 *N* NH_4OH and was purified in the same manner as for the *d* enantiomorph. After the material had been dried *in vacuo* over H_2SO_4 , it decomposed at $210-212^\circ$. A 1 per cent solution of the compound in 1 *N* NaOH gave a rotation of $[\alpha]_D^{21} = +26^\circ$. The nitrogen content of 4.76 per cent agreed closely with the theoretical content of 4.82 per cent.

Phenyl-d-cysteine—The procedure for the preparation of this compound paralleled that used in making the analogous *p*-bromophenyl-*d*-cysteine (4). The synthesis of phenyl-*l*-cysteine by the application of this procedure has been reported in full by Zbarsky and Young (9). Our product possessed a rotation of $[\alpha]_D^{22} = -70.5^\circ$ for a 1 per cent solution in 1 *N* HCl. Clarke and Inoué (10) have reported a comparable rotation in their preparation of the *l* isomer, $[\alpha]_{546}^{23.5} = +82^\circ$ for a 6.2 per cent solution in 1 *N* HCl.

p-Bromobenzyl-*d*-homocysteine—This compound was prepared from benzyl-*d*-homocysteine in the same manner as the *o*-bromobenzyl-*d*-cysteine already described in this paper. The compound possessed a rotation of $[\alpha]_D^{17} = +9^\circ$ for a 1 per cent solution in 1 *N* NaOH.

Acetyl Derivatives—The acetyl derivatives of both the *d* and *l* amino acids were prepared as previously described (1). The identity of each product was checked by nitrogen determinations. The derivatives possessed the properties shown in Table I.

Feeding Experiments

The data from the feeding experiments are summarized in Table I. The animals showed individual variation in their acceptance of the food which contained 1 to 2 per cent of the test compounds. In cases of refusal to eat the stock diet mixture (Diet I), a liquid diet (No. II) was administered by stomach tube (3). The casein diet (No. III) was found to be tolerated by the majority of the animals and was used in the later experiments as indicated in Table I. The three types of diet used were all adjudged to be nutritionally adequate.

In our earlier experiments the acetyl derivatives were isolated from the acidified urines by means of ether extraction followed by distillation of the

ether and crystallization of the residue from alcohol. Better yields were obtained by the following procedure which was adopted in experiments based on the casein diet (No. III) described above.

The total urine and washings were heated to boiling and filtered through a pad of glass wool. Hydrochloric acid was added until the solution was acid.

TABLE I
Characteristics of Products Isolated

Compound fed		Diet No.	Acetyl derivative isolated			Synthetic N acetyl-d derivative	
				M p	$[\alpha]_D$	M p	$[\alpha]_D$
	mg		mg	C	degrees	C	degrees
<i>o</i> Bromobenzyl <i>d</i> cysteine	1000	III	210	138	0	134-135	+38
<i>p</i> Bromobenzyl <i>d</i> cysteine	500	II	90	148-149	+20	142-143†	+40
	1000	I	130	142-143	+23		
	910	III	280	143-145	+19		
	960	"	300	145-146	+21		
	900	"	181		+25		
	910	"	282		+16		
	960	"	331		+20		
Phenyl <i>d</i> cysteine	1000	II	240	142-143	-17	142-143	+20
Benzyl <i>d</i> cysteine	960	III	170		-7	143-144	+42
	910	"	190		-10		
	1050	"	260		-11		
<i>p</i> Bromobenzyl <i>d</i> homocysteine	2790	II	110	139-140	+3‡	140	-2
	1000	III	170	139-141	+3‡		
N Acetylbenzyl <i>d</i> cysteine	620	"	220	143	+43	143-144	+42
	700	"	300	143	+43		+42

* Diet I, Bal Ra dog chow, *ad libitum*, Diet II, skim milk powder 25.0, Harris yeast 1.3, sucrose 31.5, Mazola 2.5, cod liver oil 4.5, Osborne Mendel salt mixture (11) 0.3, water 34.9, administered by stomach tube (3), Diet III, casein 8.0, dextrin 41.0, sucrose 15.0, Mazola 30.0, Osborne Mendel salt mixture 4.0, agar 2.0, *ad libitum*, vitamin supplementation administered in the same manner as in earlier work (12) plus 50 mg of choline chloride daily.

† Stekol (6) has reported a melting point of 118-119° for N acetyl *p* bromobenzyl *l* cysteine.

‡ The acetyl derivative was hydrolyzed to the amino acid which had the rotation of pure *p* bromobenzyl *l* homocysteine.

to Congo red. After the acidified solution had been heated for 10 minutes at 90-95°, it was treated with a small amount of decolorizing carbon and filtered while hot. The cooled solution was then extracted four times with 1 volume portions of ethyl acetate containing 1 per cent methanol. The combined ethyl acetate extracts were concentrated to dryness and the residue was dissolved in dilute ammonia. The solution was then centrifuged to remove insoluble material and acidified with concentrated

HCl Some scratching of the flask and cooling of the solution initiated crystallization, which was allowed to continue for 3 to 4 days at 0°. Alcoholic solutions were avoided in the recrystallization of the derivatives to prevent fractionation of the optical isomers. Purification was continued only until the derivatives had the correct nitrogen content. The amounts of the isolated products obtained and their characteristics are listed in Table I.

SUMMARY

Phenyl-*d*- and benzyl-*d*-cysteines are converted *in vivo* for the most part to the corresponding N-acetyl-*l*-amino acids. Some direct acetylation also takes place. The substitution of a bromine atom in the aromatic ring increased the amount of direct acetylation. *p*-Bromobenzyl-*d*-homocysteine was completely inverted. No inversion of the *l*-amino acids or of the N-acetylbenzyl-*d*-cysteine was observed.

The authors wish to thank Dr. J. R. Rachele of this laboratory for carrying out the microanalyses.

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A STUDY OF THE SYNTHESIS OF β -ALANINE IN THE WHITE RAT*

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Since the discovery of β -alanine as a constituent of the muscle extractive carnosine (1), the question of whether β -alanine is an essential dietary factor has remained unanswered. It was not until the components of the vitamin B complex were sufficiently elucidated to permit the use of synthetic vitamins in the place of crude extracts and a suitable method for the determination of β -alanine was available, that it was possible to conduct an experiment to determine directly whether the rat is capable of synthesizing β -alanine.

The experiments of Rose (2) showed that rats could be grown on diets in which purified amino acid mixtures containing no β -alanine replaced the protein component. However, the finding of Mueller and Cohen (3) that liver extract contains β -alanine led to the suspicion that perhaps other vitamin B supplement preparations might contribute β -alanine to the diet. The discovery that pantothenic acid is a β -alanine derivative (4) and that it is an essential factor for growth of the white rat (5) indicated that all adequate diets must contain β -alanine in so far as it is a part of pantothenic acid. Whether the amount which must be supplied as pantothenic acid is adequate to account for the β -alanine laid down as carnosine and anserine in muscle tissue of growing rats has not been ascertained.

In order to determine whether the white rat is capable of synthesizing β -alanine, the following series of experiments was undertaken. Two litters of rats were divided into three groups. One group was sacrificed at the beginning of the experiment and the carcasses were assayed for β -alanine. The second group was placed on a diet in which pantothenic acid was maintained at a minimum level for satisfactory growth, and the third group received a higher level of pantothenic acid and also 1 mg. of free β -alanine per day. At the end of the experimental period, these rats were sacrificed and the β -alanine content of the carcasses determined. The results are summarized in Table I.

It can be seen that the β -alanine content of both groups of rat carcasses

* The experimental work reported in this paper was carried out during the period of 1938-41. The presentation of the work has been delayed by the present emergency.

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increased about 20-fold. When the only source of β -alanine in the diet was a low level of pantothenic acid, the amount of β -alanine in the liver decreased. The growth curves of these rats also indicated that the pantothenic acid level was suboptimal. In the group supplied with a higher level of pantothenic acid, as well as with a supplement of free β -alanine, the livers showed an increase in β -alanine during the growth period. This small increase could be accounted for as storage of some of the pantothenic

TABLE I

Comparison of β -Alanine Content of Rats Grown on Diets with Low and High β -Alanine Content

Rat No. and sex	Change in body weight Initial to final	Duration of experiment	Total food consumption	Total β -alanine in diet	β -Alanine content of tissues	
					Liver	Carcass (except liver)
	gm	days	gm	mg	mg	mg
623 ♂	34	0	0	0	0.2	2
624 ♂	31	0	0	0	0.1	1
625 ♂	34	0	0	0	0.2	2
631 ♀	30	0	0	0	0.2	1
632 ♀	39	0	0	0	0.4	2
633 ♀	38	0	0	0	0.2	2
618 ♀	31-144	53	471	0.60	0.1	40
619 ♂ *	33-158	53	428	0.45	0.1	30
620 ♂	31-172	53	385	0.60	0.2	45
626 ♀	36-161	53	429	0.60	0.1	33
627 ♀	36-147	53	469	0.60	0.1	35
628 ♀	36-161	50	470	52.0	0.5	29
629 ♀	37-171	48	441	49.9	0.2	38
630 ♀	37-148	50	477	52.0	0.4	40
621 ♂	32-199	46	489	47.7	0.4	50
622 ♂	32-225	48	493	49.9	0.6	45

* Rat 619 was given no pantothenic acid until it began to lose weight from the deficiency. Added pantothenic acid cured the symptoms and growth was resumed immediately.

acid fed. The β -alanine content of the rest of the carcass increased about 30 or 40 mg regardless of the amount of β -alanine in the diet. This increase is about 50 times the amount of β -alanine supplied in the diet when the lower level of pantothenic acid was fed, and, therefore, constitutes proof that β -alanine can be synthesized by the growing white rat.

For the determination of β -alanine in the tissue of rats, the yeast growth response due to β -alanine was used. A method based on this effect was devised and is reported in this paper. This method is more rapid than the

diphtheria bacillus method described previously (6) and gives the same results with hydrolyzed carcasses. Recently, Pollack (7) has also described a yeast growth method for the determination of β -alanine. Our yeast growth method was tested with various levels of synthetic β -alanine added to suboptimal levels of tissue hydrolysates to determine whether the hydrolysates contain anything that enhances or inhibits the growth response of the yeast to β -alanine. In hydrolysates of liver tissue, where the β -alanine content was unusually low, the yeast growth method gave lower results than the diphtheria bacillus growth method. This is presumably due to the presence of toxic materials, since in these cases low dosage levels gave higher assays than did high dosage levels.

Hydrolyzed tissues were used on the one hand because of the difficulty of extracting β -alanine and its compounds completely, and on the other hand because some β -alanine derivatives do not produce the same growth stimulation as free β -alanine. Pantothenic acid under the conditions of the present study was considerably more active than free β -alanine, carnosine was about one-third as active, and anserine was inactive. All errors in analysis for β -alanine due to the inequalities in activity of these β -alanine compounds were avoided by the use of hydrolyzed tissues in the assay procedure.

EXPERIMENTAL

Feeding Experiment—Sixteen rats, at the age of 19 days, were divided into three groups. The first group, including Rats 623, 624, 625, 631, 632, and 633, was sacrificed at the beginning of the experiment. The rats of the other two groups were placed in individual cages and given food *ad libitum*. The diet contained the following ingredients per 100 gm: sucrose 63.7 gm, casein 22.3 gm, Osborne-Mendel salt mixture (8) 4.0 gm, hydrogenated vegetable oil (Crisco) 9.0 gm, corn oil (Mazola) 1.0 gm, vitamin A 4000 units, vitamin D 400 units, α -tocopherol 0.1 mg, 2-methyl-1,4-naphthoquinone 0.1 mg. In addition, the second group of rats, including Rats 618, 619, 620, 626, and 627, received daily aqueous Vitamin Supplement I and the third group of rats, including Rats 628, 629, 630, 621, and 622, received daily aqueous Vitamin Supplements I and II. The daily aqueous Vitamin Supplement I consisted of thiamine 0.02 mg, riboflavin 0.02 mg, pyridoxine 0.02 mg, nicotinic acid 0.02 mg, choline chloride 25 mg, inositol 5 mg, calcium *dl*-pantothenate 0.015 mg for the first 26 days, calcium *d*-pantothenate 0.015 mg from the 27th to the 31st day, calcium *d*-pantothenate 0.03 mg from the 31st to the 37th day, and calcium *d*-pantothenate 0.06 mg for the remainder of the experiment. The pantothenic acid content of Vitamin Supplement I was increased several times, as it became apparent from the growth curves that the level was inade-

quate The daily Vitamin Supplement II consisted of β alanine 1 mg and calcium *dl*-pantothenate 0.15 mg The change in weight, the duration of the experiment, the total food consumption, and total β -alanine ingestion for each rat are given in Table I

Preparation of Rat Carcasses for Assay—The rats were killed with ether and the gastrointestinal tract was removed and cleaned The liver was hydrolyzed separately from the rest of the carcass The carcass was ground with a meat chopper and was placed in about 500 ml of 20 per cent hydrochloric acid The mixture was boiled for about 20 hours and was filtered The excess acid was removed from the filtrate by distillation under reduced pressure The residue was dissolved in about 300 ml of water and the solution was made alkaline with sodium hydroxide The voluminous

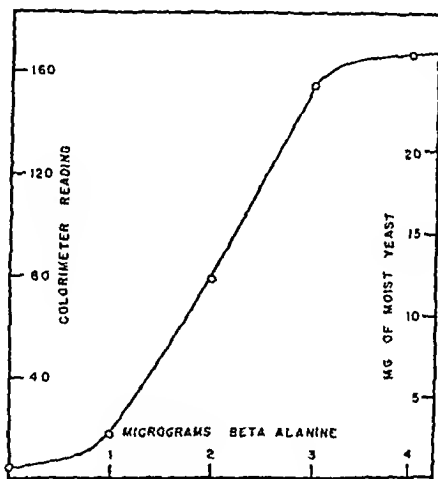


FIG 1 Effect of β alanine on yeast growth (16 hours)

precipitate was separated by centrifugation and was washed several times The solution was finally neutralized and was diluted to a convenient volume (usually 750 ml for adult rats), and then stored in a refrigerator until assayed

Yeast Growth Assay Method—The assay of β alanine with yeast is based upon the measurement of yeast growth after 16 hours of incubation A modification of a previously described medium (9) was used It contained the following ingredients per liter: sucrose (c.p.) 20 gm, ammonium sulfate 3 gm, *l*-aspartic acid 100 mg, inositol 5 mg, thiamine 0.02 mg, pyridoxine 0.02 mg, biotin 0.04 mg (live concentrate of vitamin H or crystalline biotin), KH_2PO_4 2 gm, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.25 gm, $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ 0.15 mg ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25 mg), H_3BO_3 1 mg, ZnSO_4 1 mg, MnCl_2 1 mg,

TiCl_3 1 mg, FeCl_3 0.5 mg, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.1 mg, and KI 0.1 mg. The aqueous medium containing all the ingredients except thiamine was autoclaved at 15 pounds pressure for 30 minutes. An aqueous solution of thiamine was sterilized separately with live steam for 10 minutes and was added after the rest of the medium had been autoclaved. The medium was withdrawn from this sterile flask when needed.

The aliquots to be tested (estimated to contain 1 to 3 γ of β -alanine) were made faintly acid (pH 4 to 7) and diluted to 2 ml in 50 ml Erlenmeyer flasks. The flasks were then sterilized with steam for 10 minutes. *Saccharomyces cerevisiae*, Fleischmann's Strain 139, was carried with daily transfers on wort agar slants. For inoculation, a suspension was prepared in sterile water and its concentration determined with a photoelectric colorimeter. A known amount of this preparation was added to the medium drawn from the stock flask to obtain a suspension containing 0.6 mg of yeast per 100 ml. 10 ml of the latter were then introduced into each Erlenmeyer flask and incubated at 30° for 16 hours.

At the end of the incubation period, the flasks were shaken to form a uniform suspension and the turbidity determined in a Klett-Summerson photoelectric colorimeter with a blue filter (No. 42, maximum transmission at 4200 Å). A standard curve was prepared from turbidity measurements obtained with media containing 0, 1, 2, 3, and 4 γ of β -alanine respectively (Fig. 1). The values obtained by this method for the β -alanine content of the liver and carcass of the rats are shown in Table I.

SUMMARY

It has been shown that growing rats are able to synthesize β -alanine. Rats on a low pantothenic acid diet deposited more than 50 times as much β -alanine in their tissues as was supplied in the diet. The β -alanine content of the liver tissue reflected the amount of pantothenic acid supplied in the diet, but the β -alanine content of the extrahepatic tissues was independent of dietary β -alanine. The β -alanine content of the tissues was determined by a yeast growth method which is described in detail.

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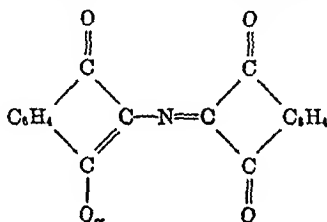
DETERMINATION OF AMMONIA EVOLVED FROM α -AMINO ACIDS BY NINHYDRIN

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Ammonia is evolved from the primary α -amino groups of amino acids when they are heated with excess ninhydrin in boiling aqueous solution. The reaction is complete in a few minutes, but the ammonia yield is quantitative only when the pH of the solution is 1.0 to 2.5. If the pH is higher, part of the ammonia condenses to form the blue anion of diketohydrindylidene-diketohydrindamine (Δ^2 -2,2'-N-bisindandione) (1-3)



The formula of the anion is given, because the blue color appears to depend on ionization.

Even at pH 1 to 2.5 the yield of ammonia from some of the amino acids, though constant, is incomplete. Therefore the present method is less accurate for determination of free amino acids in biological mixtures than is the ninhydrin- CO_2 method of Van Slyke, Dillon, MacFadyen, and Hamilton (4). Nevertheless, the two methods can be combined to advantage in order to determine the structure of a compound containing an amino acid, as was done by Folch and Schneider (5) in isolating phosphatidyl serine.

The two methods can also be combined to determine certain amino acids in mixtures with others. Whereas ninhydrin acts on proline and hydroxyproline with evolution of carbon dioxide from their carboxyl groups, their amino groups yield no ammonia, hence the difference, CO_2 minus NH_3 , may be used as a measure of proline and hydroxyproline in some amino acid mixtures. Aspartic acid also is measured differently in the two methods, yielding 2 moles of carbon dioxide and 1 mole of ammonia. In the foregoing respects the ninhydrin- NH_3 method is similar to the nitrous acid method of Van Slyke for amino nitrogen (6).

With certain other types of amines, however, the two methods differ in specificity. Unlike the nitrous acid method, with the present method only

primary α -amino groups of free amino acids are determined. For example, while nitrous acid evolves nitrogen quantitatively from β alanine, α glucosamine, and both amino groups of lysine, ninhydrin acting on these substances forms ammonia only from the α -amino group of lysine.

The main steps in the present method are, consecutively (1) the ninhydrin reaction is carried out at pH 2.5, (2) the ninhydrin is removed as an insoluble derivative, hydrindantin, (3) the generated ammonia is transferred to an acid solution by the aeration procedure of Van Slyke and Cullen (7), and (4) ammonia in the acid solution is titrated or measured manometrically by means of the hypobromite method of Van Slyke and Kugel (8).

The low pH, 2.5, of step (1), and the removal of ninhydrin in step (2), before alkalization, forestall the combination of ammonia with ninhydrin.

If the blue ninhydrin-ammonia compound were permitted to form, the yield of ammonia would be lowered. The inverse relation of blue color to yield of ammonia is shown by the following observations: (a) When the ninhydrin reaction is carried out at pH 2.5, the solution shows only a transient blue color changing to pale yellow. If, without removal of the excess ninhydrin, the pale yellow solution is mixed with alkaline hypobromite, however, the color turns to blue, and the yield of N₂ gas is variable and never exceeds 0.6 of the α -nitrogen of the amino acid. (b) If the present method is followed in every detail except that buffering at pH 4.7 is substituted for buffering at pH 2.5, a persistent blue color forms and the yield of ammonia is variable and less than 0.8 mole per mole of amino acid. (c) If the pH during the reaction is at 1.0 to 2.5 and the ninhydrin is then removed in step (2), the solution shows no persistent blue color during the ninhydrin reaction or during the liberation of ammonia by addition of alkali in step (3), and the yield of ammonia is quantitative or constant.

Complete precipitation of ninhydrin from aqueous solution can be accomplished in either of two ways described by Ruhemann (1), by reduction of ninhydrin to hydrindantin by means of hydrogen sulfide, $2C_9H_5O_3 + H_2O + H_2S = C_{18}H_{10}O_6 + S + 2H_2O$, or by condensation of ninhydrin with *o*-phenylenediamine to form a diketophenazine compound. Both hydrindantin and the diketophenazine compound are insoluble at pH 2.5, but hydrogen sulfide is the more convenient reagent.

Apparatus

For the Ninhydrin Reaction—The all-glass reaction vessel of Hamilton and Van Slyke (9) (Fig. 1, A).

For Determination of Evolved Ammonia—The apparatus for determination of ammonia by aeration, described by Van Slyke and Cullen (7). A flow meter is useful to measure the air current during aeration, in order to make

sure that at least 75 liters of air are carried through the solutions. When ammonia is measured manometrically, the Van Slyke-Neill apparatus is needed.

Reagents

Besides those reagents needed for the ninhydrin reaction (4), the present method requires hydrogen sulfide and the reagents described for the determination of ammonia by aeration (7). For manometric measurement of ammonia the hypobromite reagent (8) is used. For titration of the ammonia 4 per cent boric acid solution (10) and $\sqrt{70}$ sulfuric acid (7) are employed.

Procedure A Measurement of NH_3 Formed by Action of Ninhydrin

The ninhydrin reaction is carried out in aqueous solution at pH 2.5, according to the method of Van Slyke, Dillon, MacFadyen, and Hamilton ((4) pp 639-646), preferably with amounts of amino acid yielding 1 to 1.5 mg of ammonia nitrogen. (The results to be presented were obtained from analysis of reaction solutions of 1 ml volume, containing 50 mg of ninhydrin and from 50 to 100 mg of citrate buffer mixture at pH 2.5. As previously directed ((4) p 643), the reaction time was 8 minutes, unless otherwise noted in the experimental part of the present paper.)

After evolution of ammonia and carbon dioxide, the CO_2 and traces of air are removed from the reaction vessel by means of suction for 2 minutes. Before the suction tube is disconnected, the evacuated reaction vessel is closed by a turn of its stopper. Then H_2S is admitted to the vessel till atmospheric pressure is restored. The vessel is closed again by a turn of its stopper, the generator is disconnected, and the solution is saturated with hydrogen sulfide and the hydrindantin is precipitated by shaking vigorously for 4 to 5 minutes. The excess H_2S is then removed from the vessel by applying suction for 4 minutes or longer.

The H_2S -free mixture is transferred to a 15 ml volumetric flask, three washings with citrate buffer solution (50 mg of the citrate mixture of pH 2.5 in each ml of water) being used to complete the transfer. The mixture in the flask is diluted with water to a volume of 15 ml, mixed, and then filtered from the hydrindantin precipitate. 10 ml of filtrate are taken for ammonia determination by the aeration method of Van Slyke and Cullen (7). Either the aerated ammonia is absorbed in 25 ml of 4 per cent boric acid solution and is measured by titration with $\sqrt{70}$ sulfuric acid, with a final volume of 50 ml at the end-point ((11) pp 689-690), or the ammonia is absorbed in 12 ml of 0.1 N sulfuric acid and measured manometrically in the manner described by Van Slyke, Hiller, and MacFadyen ((11) pp

687-688) All the results presented in the experimental part of this paper were obtained manometrically

Procedure B Measurement of Both the NH₃ and the CO₂ Formed by the Action of Ninhydrin

The reaction is the same as in Procedure A, except that a smaller sample is taken, enough to contain only 0.4 to 0.6 mg. of carboxyl nitrogen, in order to keep the CO₂ yield within the limits measurable at 2 ml. volume in the manometric apparatus. After the reaction with ninhydrin the CO₂ is measured as in the ninhydrin-CO₂ method (4). The reaction vessel is then evacuated, filled with hydrogen sulfide, and the rest of the procedure for the NH₃ determination is carried through as in Procedure A.

EXPERIMENTAL

Yields of Ammonia from Amino Acids and Other Substances

The results are shown in Table I. Carboxyl nitrogen was measured by the ninhydrin-CO₂ method (4), amino nitrogen by the nitrous acid method (6), and ammonia nitrogen by the present method. COOH-N and NH₃-N in the same sample of material were measured in sequence as described in Procedure B, a different sample was used for the measurement of amino nitrogen.

Noteworthy results are the failures of β -alanine, α -glucosamine, urea, and hydroxyproline to yield ammonia. Among the amino acids tested, glycine and α -alanine gave less than theoretical yields of ammonia, and the yield from tryptophane was surprisingly low. Of interest is the fact that lysine by reaction with ninhydrin evolves ammonia (as well as carbon dioxide) beyond 1 mole when the boiling period at pH 1.0 exceeds 10 minutes, in 30 minutes the yield was 1.03 moles.

The constancy of yield of ammonia from α -alanine under varied conditions is shown in Table II. In no instance did the addition of β -alanine improve the yield, though analogous use of glycine and β -alanine, for the purpose of preventing combination of ammonia with nascent aldehyde, had been effective in the method of Van Slyke, Hiller, and MacFadyen ((11) p. 682) for the determination of hydroxyamino acids. Lowering the pH from 2.5 to 1.0 and prolonging the time of reaction also failed to change the yield of ammonia from α -alanine.

Substances Showing Primary Amino Nitrogen Remaining after the Ninhydrin Reaction at pH 2.5

Primary amino nitrogen, in solution after the ninhydrin reaction, was estimated by measuring the nitrogen evolved by the nitrous acid reaction.

TABLE I

Determination of Amino Groups by Nitrous Acid, Ninhydrin CO₂, and Ninhydrin NH₃ Methods

Substance	N determined per mole substance		
	NH ₂ -N by nitrous acid reaction (6)	COOH N by ninhydrin CO ₂ reaction (4)	NH ₂ N by ninhydrin NH ₃ reaction
	gm atoms	gm atoms	gm atoms
Glycine	1 01	0 95	0 86
α -Alanine	1 00	1 00	0 90
Tryptophane	1 02	0 87	0 34
Glutamic acid	0 99	1 00	0 97
Aspartic "	1 02	1 97	0 98
Arginine HCl	0 99	1 00	1 00, 1 03*
Histidine 2HCl	0 99	1 00	0 99
Lysine 2HCl	1 99†	1 01†	1 00†
Hydroxyproline	0	0 98	0
β Alanine	1 00	0 001	0 001
α Glucosamine HCl	1 01	0	0
Urea	0 13	0 009	0 003

* One out of eight analyses

† The reaction time was five times as long as standard, in order to complete the evolution of nitrogen from the ϵ amino group

‡ Ninhydrin reaction at pH 1 for 10 minutes

TABLE II

Constancy of Yield of Ammonia from α Alanine by Reaction with Ninhydrin under Varied Experimental Conditions

Ninhydrin reaction			Amino N recovered as ammonia
pH	Time	Remarks	
	min		per cent
2 5	10	20 mg β alanine added before reaction	91 6, 90 8, 90 6
2 5	10	No β alanine added	90 6, 90 5, 89 6
2 5	10	20 mg β alanine added after reaction but before determination of ammonia	90 8, 90 1, 88 9
2 5	6		89 3
2 5	15		90 0
2 5	20		89 8
1 0	12		89 0
1 0	18		90 8
1 0	24		89 3

A correction for nitrogen evolved by nitrous acid from ammonia present was made, it was found that ammonia, after subjection to the ninhydrin

THE USE OF LOW ENVIRONMENTAL TEMPERATURE DURING THE PREPARATION OF TISSUE SLICES FOR RESPIRATION STUDIES IN VITRO*

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The methods commonly used for the preparation of tissue slices have, in many instances, involved the maintenance of the tissue at body temperature during preparation (1-4). In the course of investigations on the effect of low environmental temperatures on the metabolism of mammalian tissues it occurred to us that instead of attempting to maintain the tissue at body temperature a more rational procedure would be the preparation of tissue slices at low temperatures. The use of low temperature in this case is based upon the hypothesis that metabolic processes should be reduced to a low level during the time when the oxygen supply is inadequate to meet the demands of the tissue. Such a condition of inadequate supply prevails upon separation of an organ from its blood supply, and before slices of the tissue are placed in an oxygenated medium. This paper presents data on the oxygen consumption of rat liver, cerebral cortex, and kidney cortex slices at 37° after these tissues have remained for various lengths of time in moist chambers at 10° and at 35°. The advantage of the use of low temperature during the preparation of kidney and liver slices is apparent from the data presented.

Methods

Preparation of Slices and Measurement of Oxygen Consumption—Tissues from eleven well fed, adult albino rats weighing from 200 to 250 gm. were used. The animals were decapitated and the organs to be used removed as rapidly as possible. One cerebral hemisphere, one lobe of liver, or one kidney was transferred to a moist chamber maintained at 10°, and the remaining tissue was placed in a moist chamber at 35°. The tissues were kept in covered Petri dishes, humidified with moist filter paper on the cover, during the period that they remained in the chambers. Two slices of each organ in each chamber were made immediately, two after 30 minutes, and two more after 60 minutes. These were then weighed rapidly on a micro torsion balance and placed in respirometer vessels. The medium was Ringer's solution (5), buffered to pH 7.25 with 0.01 M phosphate and

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containing 0.011 M glucose. The gas phase was oxygen. Oxygen consumption was determined by the direct method of Warburg in a thermostat at 37.7°. Rates of oxygen consumption are expressed as cmm of O_2 consumed per mg of initial dry weight (determined on aliquots) per hour (QO_2). The preparation of brain slices has been described previously (4). Slices of kidney cortex were prepared with a modified Terry microtome (6), and slices of liver with the instrument described by Martin (7).

Construction of Moist Chambers—The cold moist chamber was a rectangular glass and wood box approximately 24 inches deep, 30 inches wide,

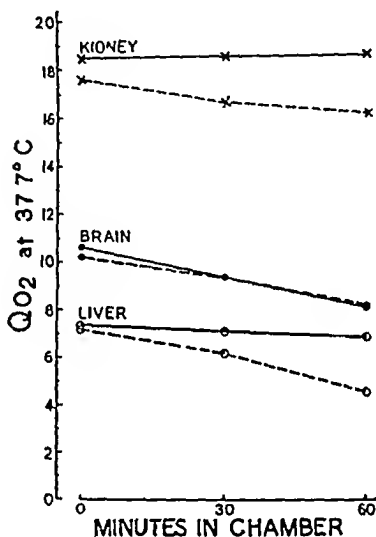


FIG 1 Oxygen consumption of slices of rat kidney cortex, cerebral cortex, and liver at 37.7° following preparation and maintenance in warm and cold chambers at 10° and 35°. Solid lines, tissue prepared and maintained at 10°, broken lines, tissue prepared and maintained at 35°.

15 inches high in the back, and 10 inches high in front. The sloping top was of glass. The front was designed to admit the hands through holes covered with rubber flaps. The whole box was well illuminated by fluorescent lamps placed behind a frosted glass plate which formed the back of the box. The chamber was cooled by a refrigerated coil placed at the top and back of the box. The atmosphere was kept nearly saturated by means of cold distilled water which dripped over a gauze wick through which air was circulated by a small fan. A heating coil and thermostat were included so that a definite temperature could be maintained. The warm chamber was similarly constructed except that heating and saturation

tion of the atmosphere were attained by means of a beaker of water on a thermostatically controlled electric plate (*cf* (8))

Results

A comparison of the oxygen consumption of tissue slices made as soon as possible and after 30 and 60 minutes respectively in the cold and warm chambers is shown in Fig 1. Each point for kidney and brain represents

TABLE I

Oxygen Consumption of Rat Liver Slices at 37.7° after Various Periods at High and Low Environmental Temperatures

Medium, Ringer phosphate glucose, pH 7.25. The liver was removed as rapidly as possible and placed in moist chambers saturated with water vapor, one of which was maintained at 10° and the other at 35°. Duplicate samples from each chamber were taken immediately and after 30 and 60 minutes for determination of QO_2 .

Chamber temperature	Animal No	QO_2 at 37.7		
		Immediate	30 min	60 min
C 10	1	7.66	7.23	6.20
	2	5.77	5.85	5.75
	3	8.03	7.57	7.61
	4	7.37	6.80	6.96
	5	8.15	8.06	7.94
Mean		7.40	7.10	6.89
35	1	7.22	6.34	4.09
	2	5.53	5.22	4.40
	3	7.89	6.47	3.66
	4	7.32	5.34	4.90
	5	8.02	7.41	5.66
Mean		7.20	6.16	4.54
P		0.05	0.01	0.01

* Mean QO_2 of duplicate samples from one animal

the mean QO_2 of duplicate samples from three animals, while each point for liver represents the mean QO_2 of duplicate samples from five animals. It is clear from Fig 1 that the QO_2 at 37.7° of kidney cortex and liver slices was lower if they were maintained near body temperature before being sliced. In contrast, if the tissue was maintained for 60 minutes in the chamber at 10°, the subsequently determined QO_2 in the case of kidney was not lower than the initial rate and only about 7 per cent lower in the case

of liver The final QO_2 of brain, however, decreased on standing in either chamber Under these conditions the rates of oxygen consumption observed after varying periods in either chamber were steady states for all three tissues

The data for liver are given in Table I The difference between the QO_2 of liver sliced immediately in the warm and cold chambers may be significant ($P = 0.05$), while the difference after 30 or 60 minutes is significant ($P = 0.01$) (method of paired differences (9)) The data for kidney were not sufficient to permit statistical analysis, but it appears that the differences shown in Fig. 1 for this tissue are also reliable

Determinations of water content were made on aliquots of liver which had remained in each moist chamber for 60 minutes The mean percentage dry weight of liver from the cold chamber was 29.15 (duplicate samples from five animals), while that from the warm chamber was 28.91 per cent The difference between these mean values is not significant ($P = 0.1$)

DISCUSSION

When tissues from homothermal animals were first studied *in vitro*, some investigators were concerned with the consequences of the sudden reduction of temperature of the tissue upon removal from the body, and before transfer to a thermostat at body temperature This was perhaps first clearly stated by Bass (10) We have, however, shown that slices of rat brain and kidney cortex can be cooled rapidly to 0° , maintained at this level for 1 hour, and returned to 37.7° without loss of respiratory or glycolytic capacity (11) It thus appears that sudden cooling produces no tissue injury, if capacity for respiration and glycolysis be the criteria, provided that the temperature remains above 0° (cf. (12-14)) The results presented here indicate that the respiratory capacity of liver and kidney tissue is in fact better maintained if the tissue is cooled during the period in which the oxygen supply is inadequate

In the case of brain the decrease in QO_2 was no more marked in the warm chamber than in the cold chamber A possible explanation for this may be found in the low carbohydrate content of nervous tissue (15), and in its dependence on carbohydrate for substrate (cf. (16)) The stored carbohydrate supply may be exhausted during the time required for removal of the brain and for it to attain the temperature of the cold chamber Glycolysis could not occur under these conditions because of lack of substrate, and respiration is limited by insufficient oxygen supply The lack of energy-yielding reactions in the tissue may then result in irreversible damage to the respiratory mechanisms This is in agreement with the data of Warburg, Wind, and Negelein (17) who reported that in order

to destroy tumor cells through lack of energy it is necessary to deprive them of both glucose and oxygen. In the case of liver and kidney, carbohydrate storage and the ability to metabolize non-carbohydrate substrate may be sufficient to account for the better maintenance of QO_2 at 10° , whereas these mechanisms may be inadequate at 35° . Unpublished work in this laboratory has shown that there is irreversible decrease in the capacity for respiration when brain slices are exposed to temperatures higher than 41° , even though the oxygen supply is adequate. Such irreversible changes, however, do not occur at 35° if the oxygen supply is maintained.

Experiments in which viability of tissue has been determined after maintenance at high and low environmental temperatures (*cf* (18, 19)) have demonstrated the advantage of low temperature under different conditions from those reported here. Recent investigations have demonstrated the better survival of ischemic tissues at low environmental temperatures (20-22). The results obtained in this investigation are entirely consistent with the view that, under circumstances in which oxygen supply to a tissue is inadequate to meet the metabolic demands of the tissue, injury is prevented by a reduction in tissue metabolism, which may be accomplished by a reduction in temperature.

SUMMARY

The oxygen consumption of slices of rat cerebral cortex, kidney cortex, and liver was determined at 37.7° after the tissue had been prepared in moist chambers maintained at 10° and at 35° . In the case of liver and kidney the QO_2 of slices taken as soon as possible and after 30 and 60 minutes was lower if the tissue was kept in the warm chamber than if it was kept in the cold chamber. In the case of brain the QO_2 was not influenced by the temperature of the chamber in which it was maintained and prepared. The data for liver and kidney are consistent with the hypothesis that under conditions of inadequate oxygen supply tissue injury is prevented by a reduction of the metabolism of the tissue through a reduction in temperature.

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POTASSIUM AND CALCIUM IN EPIDERMAL CARCINOGENESIS INDUCED BY METHYLCHOLANTHRENE*

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In our integrated program on epidermal carcinogenesis in mice, due to methylcholanthrene, studies on the effect of methylcholanthrene upon the fixed alkalies, ascorbic acid, and iron of normal, benzene-treated, and methylcholanthrene-treated epidermis have been reported (1). These investigations showed that a single application of the carcinogen reduced within 10 days the epidermal iron and calcium content to 50 per cent of the normal. Multiple treatments of the epidermis with the carcinogen on alternate days for 60 days induced a further lowering in the iron content, but did not appreciably affect the calcium diminution produced by one application. On the other hand potassium, sodium, magnesium, and ascorbic acid were not significantly altered by a single or by prolonged treatment of the epidermis with the carcinogen. The extent to which the cytological, chemical, and physical studies in our program of epidermal carcinogenesis have been integrated is given in a résumé by Cowdry (2).

The next step was to determine the mineral content of a carcinoma derived from mouse epidermis. The chemical analysis of the tumor cells alone is beset with difficulties because of the extent of necrosis, keratinization, and because of the amount of blood present. The use of methylcholanthrene-induced (spontaneous) tumors of the skin is inadequate for analysis, because the tumors induced vary in their degree of keratinization, differentiation, and necrosis, which would make difficult the sampling of tumors of a more or less constant chemical composition. Therefore we chose to use a transplantable squamous cell carcinoma originally produced in the epidermis of a Swiss mouse by the application of methylcholanthrene.

EXPERIMENTAL

The methods for the determination of calcium (3), potassium (1), and of nucleoprotein phosphorus (4), the basis of reference, have been given. The tumors were freed from adhering blood and connective tissue and then cut into small pieces and mixed before sampling. In the case of potassium it was necessary to determine the iron content of a portion of the sample.

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to ascertain the amount of potassium in the tumor due to the presence of blood. This was unnecessary for calcium, as the estimation of the hemoglobin content of many tumors showed that blood calcium of the tumor was insignificant.

The authors are indebted to Dr. Z. Cooper¹ for the carcinoma which had passed through fifteen transplants. During the time in which our chemical analyses have been undertaken the carcinoma has passed through ten more transplants and its characteristics have remained practically constant, being a differentiated squamous cell carcinoma with pearl formation. By using a portion of the tumor sample for the determination of iron, it is easy to estimate the percentage of a particular metal in the tumor due to the presence of blood. The effect of necrosis on the mineral composition of tumors has long been known (5), and the importance of rigorously excluding this material from tumors in order to obtain the composition of tumor cells alone is discussed below and summarized in Table I.

Calcium—The results for the calcium analyses are expressed as mg of calcium and mg of nucleoprotein phosphorus (NPP) per 100 mg of tissue, and by the ratio $\text{Ca NPP} \times 10$. The calcium content of large tumors, freed of all gross necrotic material, varied from 0.038 to 0.045 mg per 100 mg of tumor (average 0.042), and the Ca NPP ratio was 3.42. Then smaller tumors (about 15 mm in diameter) which were cleaned of necrotic tissue visible to the naked eye were employed. The calcium content varied from 0.025 to 0.033 mg per 100 mg (average 0.029), with a Ca NPP ratio of 2.42. Still smaller tumors (about 10 mm in diameter) had a calcium content of 0.015 to 0.024 mg per 100 mg (average 0.019) and a Ca NPP ratio of 1.58, which was about the same as that of hyperplastic epidermis. A small piece of each tumor analyzed was fixed and examined microscopically for necrotic tissue to ascertain the relationship between necrosis and calcium content. The latter increased with the amount of degenerating tissue. Even the smaller tumors showed foci of necrosis, although they appeared grossly quite solid after removal. To test the relationship between the calcium content and necrosis further, large necrotic tumors were analyzed and were found to be very rich (0.077 mg per 100 mg) in this metal, an observation noted by many (5) previous investigators.

Since the calcium content varied directly with the amount of necrotic tissue, and since minute amounts of the latter considerably affected the calcium content, very small tumors (5 to 8 mm in diameter) were employed. These were solid, and microscopically showed little, if any, necrotic residues. Eight to twelve tumors were used for a single analysis. The calcium content varied from 0.007 to 0.013 mg per 100 mg (average

¹ Cooper, Z. K., Ferminger, H. I., and Reller, H. C., manuscript in preparation.

0.009) of tumor, and had a Ca NPP ratio of 0.75 (Table II). This was about 50 per cent less than that of hyperplastic epidermis, which was again 50 per cent lower than normal epidermis. In a preliminary note the

TABLE I
Effect of Necrosis upon Calcium-Nucleoprotein Phosphorus Ratio of Carcinoma

Carcinoma	No of analyses	Ca per 100 mg		NPP per 100 mg average	Ca NPP $\times 10$
		Extremes	Average		
		mg	mg	mg	
Large and necrotic " freed of all gross necrotic tissue	4	0.054-0.088	0.077	0.123	3.42
	9	0.038-0.045	0.042		
About 15 mm in diameter, freed of gross necrotic tissue, but showed latter microscopically	9	0.025-0.033	0.029	0.120	2.42
	11	0.015-0.024	0.019	0.120	1.53

TABLE II
Calcium-Nucleoprotein Phosphorus Ratio in Epidermal Methylcholanthrene Carcinogenesis

Tissue	No of analyses	Ca per 100 mg tissue	NPP per 100 mg tissue	Ca NPP $\times 10$
Normal epidermis	6	mg	mg	
Benzene-treated epidermis	7	0.044	0.118	3.70
Methylcholanthrene-treated epidermis	18	0.042	0.125	3.40
Carcinoma, solid, about 5-8 mm in diameter with little, if any, necrosis		0.019	0.130	1.46
		0.012	0.133	0.90
		0.013	0.124	1.05
		0.008	0.096	0.83
		0.008	0.096	0.83
		0.007	0.123	0.57
		0.009	0.123	0.73
		0.008	0.133	0.60
		0.007	0.133	0.52
		0.009	0.120	0.75
Average (8 analyses)				

calcium deficiency of the tumor cells alone of this carcinoma was reported (6).
Potassium—Since whole blood contains considerable amounts of potas-

sium, it is necessary to correct for the amount of this metal present in tumors as blood potassium. To do this the potassium and iron content of mouse whole blood was determined as follows. The tail of a mouse was kept in warm water for a few minutes to dilate the blood vessels, wiped dry, and the end of the tail was cut with a sharp razor blade. The drops of blood (about 100 mg) were allowed to fall into a glass-stoppered weighing bottle containing about 1 cc of distilled water (to luke the blood) which had been previously weighed. The increase in weight gave the amount of blood present. The entire contents of the weighing bottle were rinsed into a silica crucible to which was added 1 drop of concentrated H_2SO_4 . The crucible contents were heated to dryness on a steam bath and ashed completely at 450° .

Eight samples of mouse whole blood contained an average of 0.253 mg of potassium per 100 mg of blood, and eleven samples an average of 0.047 mg of iron per 100 mg. Assuming that nearly all of the iron in the tumors is from hemoglobin, from the amount of the former per 100 mg of any

TABLE III

Effect of Necrosis upon Potassium-Nucleoprotein Phosphorus Ratio of Carcinoma

Carcinoma	Average K per 100 mg	Average Fe per 100 mg	Average blood K per 100 mg	Average K per 100 mg corrected	Average NPP per 100 mg	K \ NPP
	mg	γ	mg	mg	mg	
Moderate necrosis (7 analyses)	0.286	2.0	0.011	0.276	0.132	2.09
Extensive necrosis (7 analyses)	0.275	2.0	0.011	0.264	0.133	1.91

tumor it is easy to calculate the amount of whole blood present and from the latter the potassium content to give the corrected value. Therefore on such a tumor or pool of tumors three determinations were carried out, nucleoprotein phosphorus, potassium, and iron.

Although the potassium content of the carcinoma was not affected as much by the presence of necrotic material as was the calcium, nevertheless the influence was demonstrable. Moreover the high concentration of blood potassium necessitated its determination in the carcinoma in order to get the true value for the tumor cells alone. The results for tumors with necrosis are shown in Table III, and are expressed in the same manner as for calcium except that the K NPP ratio was not multiplied by 10. The values given below were corrected for potassium in the tumors due to the presence of blood. The tumors having a moderate amount of necrosis contained 0.276 mg of K (average) per 100 mg and had a K NPP ratio of 2.09, while those with more extensive necrosis had 0.264 mg of K (average) per 100 mg and a ratio of 1.91, which was 28 per cent less than normal and

hyperplastic epidermis. The potassium content decreases and the calcium content increases with the amount of degenerative tissue present in the tumor.

The small solid tumors which showed little, if any, necrosis (Table IV) microscopically contained 0.313 mg of K per 100 mg (average) and had a K/NPP ratio of 2.24. Eight to twelve of these tumors were employed for a single analysis. The K/NPP ratio of the small tumors was about 16 per

TABLE IV
*Potassium-Nucleoprotein Phosphorus Ratio in Epidermal
Methylcholanthrene Carcinogenesis*

Tissue	No. of analyses	K per 100 mg tissue	Fe per 100 mg tissue	Blood K per 100 mg tissue	K per 100 mg tissue corrected	NPP per 100 mg tissue	K/NPP
		mg	γ	mg	mg	mg	
Normal epidermis	4	0.345				0.129	2.68
Benzene-treated epidermis	4	0.351				0.134	2.62
Methylcholanthrene-treated epidermis	19	0.346				0.130	2.66
Carcinoma, solid, about 5-8 mm in diameter with little if any, necrosis		0.363	2.3	0.012	0.351	0.149	2.35
		0.311	2.7	0.015	0.296	0.134	2.21
		0.326	2.7	0.015	0.311	0.134	2.32
		0.330	2.7	0.015	0.315	0.140	2.25
		0.336	2.7	0.015	0.321	0.140	2.29
		0.325	2.3	0.012	0.313	0.137	2.28
		0.314	2.3	0.012	0.302	0.137	2.20
		0.334	2.1	0.011	0.323	0.152	2.12
		0.324	2.3	0.012	0.312	0.141	2.21
		0.329	2.3	0.012	0.317	0.141	2.24
		0.313	1.7	0.009	0.304	0.138	2.20
		0.311	1.7	0.009	0.302	0.138	2.19
		0.335	2.5	0.013	0.322	0.143	2.25
		0.317	2.5	0.013	0.304	0.143	2.12
		0.312	2.3	0.012	0.300	0.125	2.40
Average (15 analyses)		0.325			0.313	0.139	2.25

cent less than that of normal and hyperplastic epidermis and 8 per cent higher than that of the necrotic tumors. The amount of blood potassium in all the tumors, necrotic or solid, averaged about 4 per cent of the total

DISCUSSION

These studies demonstrate that chemical analyses of tumors, at least for the minerals, are without significance unless the factors of necrosis, blood supply, and size are considered. Even when the gross necrotic tissue

of large tumors is carefully cleaned out (leaving only the viable periphery) microscopically there are sufficient foci of degenerating tissue, or penetration of the latter around the living tumor cells, to affect the calcium content considerably and to a less extent the potassium content

In a comprehensive review on the rôle of the fixed alkalis in cancer, Shear (5) stated "that much confusion exists as regards the role in cancer of the commonly occurring constituents, sodium, potassium, calcium, and magnesium." Although there was better agreement as regards the distribution of calcium and potassium in tumors, *i.e.* that rapidly growing tumors contained more potassium and less calcium, Shear pointed out that even these results could not be accepted without further investigation. The studies on these metals in the carcinoma reported in this paper demonstrate that even small foci of necrosis can affect considerably the calcium content, and to a less extent the potassium content. The latter present as blood in the tumors must be determined to obtain the true value for the tumor cells alone. Provided care is taken in the sampling of the tumors, our studies, at least for the rapidly growing carcinoma used, reveal that the tumor cells are deficient in calcium and contain less potassium than do normal and hyperplastic epidermis.

These studies show that the process of epidermal carcinogenesis occurs in two distinct phases: an immediate reduction in the calcium content of hyperplastic epidermis which persists for many weeks and a further diminution when the epithelial cells have become malignant. Although it is not possible to state the significance of the calcium changes, observations of others may lend importance to them. According to Axelrod, Swingle, and Elvehjem (7) available data indicate that succinate, succinic dehydrogenase, cytochrome *c*, cytochrome oxidase, and oxygen are obligatory components of the succinoxidase system. And since Swingle, Axelrod, and Elvehjem (8) have further demonstrated that calcium stimulates the succinoxidase system by activating the nucleotidase of tissue cozymase in such a manner that cozymase is destroyed and cannot function in the dehydration of malate to oxalacetate, this evidence would seem to indicate either that the low calcium content of tumors was insufficient to activate the cozymase nucleotidase, or that the tumors were deficient in succinic dehydrogenase. That the latter enzyme is deficient in tumor tissues has been demonstrated (9). Further study is necessary to determine the significance of the low calcium and iron content of hyperplastic epidermis. Perhaps the diminished iron content may be associated with a decrease in the amount of cytochrome *c*.

Although the micro incineration technique is not suitable for quantitative analysis or for differentiating between the white ash of calcium and magnesium, or between the bluish ash of sodium and potassium, it is useful in a

qualitative way to estimate an increase or decrease in the white ash of calcium or magnesium. It does have the advantage of permitting one to locate in the cell the position of the metals. Paletta, Cowdry, and Lischer (10) found demineralization in both benign and methylcholanthrene hyperplasia, particularly in the distal part of the spinous layer. Scott (11) also reported that hyperkeratosis, warts, and human breast and skin carcinoma showed much less calcium or magnesium in their cytoplasm than did similar normal types. Cathie (12) has postulated that an increase in the intranuclear ash content of calcium and magnesium is concomitant with marked radiosensitivity, radioresistance being associated with a decrease in the white ash.

SUMMARY

The rôle of potassium and calcium in epidermal carcinogenesis induced by methylcholanthrene is discussed. The rapidly growing transplantable carcinoma used in these studies was found to contain much less calcium than hyperplastic epidermis, which again has 50 per cent less calcium than normal epidermis. Also the potassium content of the carcinoma was about 16 per cent less than that of normal and hyperplastic epidermis. The two step decrease in the calcium content reveals that the process of epidermal carcinogenesis occurs in two distinct phases: an immediate reduction in the calcium content in hyperplastic epidermis which persists at a fairly constant level for many weeks and a further diminution when the epithelial cells have been transformed into cancer cells. The possible significance of these chemical changes is briefly summarized. Nucleoprotein phosphorus was used as a basis of reference for the amount of living tissue involved.

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STUDIES ON PYROGENS

I THE ISOLATION OF PYROGENS FROM VARIOUS MICROORGANISMS

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In the use of vaccines, of antibacterial and antitoxin preparations, of serums, plasma, and other fluids in transfusions, reactions involving high temperatures and chills have not infrequently been observed. These reactions are ascribed to substances called pyrogens, which may be produced in the growth and metabolism of certain microorganisms. In the past, the study of such bacterial pyrogens has been focused principally on their elimination from solutions used for intravenous injections and on methods of testing for their presence. The present investigation plans for the study of pyrogens as definite substances and their chemical, physical, and physiological properties and actions. This paper presents the results obtained in the purification of pyrogens from triple vaccine (*Eberthella typhosa*, *Bacillus paratyphosus* A and *Bacillus paratyphosus* B), *Pseudomonas aeruginosa* (*Bacillus pyocyaneus*), and *Proteus vulgaris*, and the pyrogenic actions on rabbits of these substances.

The existence of pyrogens from bacterial sources was made evident by Wechselmann (1) and by Hort and Penfold (2). Extensive studies by Seibert and her coworkers (3), Rademaker (4), Banks (5), and others put the problem on a firm experimental basis. The more recent work of Co Tui and his associates (6) included a careful study of the method of testing as well as methods of removing pyrogens from solution and the purification of certain pyrogen materials.

It may be stated that the study of the chemical nature of pyrogens to date has followed two distinct lines. This is indicated by the following reports. Co Tui, Hope, Schrift, and Powers (7) concentrated and purified the pyrogen from *Eberthella typhosa* and obtained a product with 1.5 per cent N, 1.25 to 1.29 per cent S, 0.61 to 0.67 per cent P, and 4.2 to 4.5 per cent ash. It was suggested that the product was of non-protein character. The careful study of Welch, Calvery, McClosky, and Price (8) tentatively followed the plan that "although there is no evidence to show that the potency of the pyrogenic substance is associated with the total nitrogen content, it seemed advisable to utilize it as an index of the strength of the pyrogenic solution."

EXPERIMENTAL

The method of carrying out the pyrogen tests on rabbits was uniform for the various products. It will therefore be outlined first and the results obtained in the testing of the products presented following the description of their purification.

Method of Pyrogen Testing—All reagents were free from pyrogens. The water used was either passed through an Ertel pyrogen-retentive filter or distilled (glass condenser) and then autoclaved. All equipment used was washed with pyrogen-free water. In the tests, suitable amounts of the material were dissolved so that 1 ml of the final solution per kilo of rabbit contained the quantity to be tested for pyrogen action in isotonic salt solution. The temperatures of the rabbits were taken before injection of the solutions intravenously through the marginal ear vein and at 1 hour intervals thereafter for 5 hours. The care of the rabbits, as well as the requirements and conditions for satisfactory testing, was similar to that employed by Welch, Calvery, McClosky, and Price (8) and McClosky, Price, Van Winkle, Welch, and Calvery (9). The rabbits weighed more than 1000 gm each.

Procedure for Isolation of Pyrogens from Proteus vulgaris, Eberthella typhosa, and Pseudomonas aeruginosa—Saline suspensions of *Proteus vulgaris* were obtained from Dr. Co. Tui, and suspensions of *Eberthella typhosa* (containing also paratyphoids A and B) and *Pseudomonas pyocyaneus* were furnished by the Lederle Laboratories.

2.5 liters of the saline suspension of organisms containing approximately 12×10^{10} organisms per ml were poured into 10 volumes of acetone, and 100 ml of glacial acetic acid added to coagulate the turbid suspension. The precipitate formed was allowed to settle and the supernatant liquid discarded. The insoluble material was dried *in vacuo* over sulfuric acid until free of acetone odor. This was necessary to prevent foaming in the next step. (If a suspension of organisms in the culture medium was used, the total solids were approximately 25 gm, owing to proteins and salts present in the medium¹). The dry powder (10 to 25 gm) was then suspended in 100 ml of water to which 3 ml of toluene had been added. This suspension was heated on the steam bath for 1 to 2 days, cooled to room temperature, placed in a Visking sausage casing with a few drops of toluene, and dialyzed against running tap water overnight. The dialyzed material was centrifuged for 2 hours at 5000 R P M in the angle centrifuge and the residue discarded. The clear supernatant liquid (approximately 130 ml) was mixed with 10 volumes of acetone and 5 ml of glacial acetic acid were

¹ In the case of dried organisms these steps can be omitted. 5 to 10 gm of dried organisms were mixed with 100 ml of distilled water to which 3 ml of toluene had been added, and the mixture heated on a steam bath for 1 to 2 days.

added. The precipitate formed was separated by centrifugation and dried *in vacuo*. The yield was approximately 2 gm. The dried precipitate was mixed with 5 ml of water, heated on the steam bath, and centrifuged to remove insoluble material.

To the clear supernatant liquid, 6 volumes of 95 per cent phenol (10) were added slowly with good stirring, so that the precipitate which formed remained finely divided. The resulting suspension was stirred intermittently for 1 day. The material insoluble in the phenol was separated in the centrifuge and the phenol extraction of the precipitate repeated several times, with 15 ml of 95 per cent phenol the second time, 5 ml the third time, etc. Four extractions were sufficient to free the *Proteus vulgaris* pyrogen and typhoid pyrogen of nitrogen compounds. However, in the case of *Pseudomonas aeruginosa*, it was necessary to wash out the phenol from the residue with acetone after the third phenol treatment. The washed residue was mixed with 3 ml of water and heated on the steam bath. Any water-insoluble material was removed by centrifugation. The clear supernatant liquid was concentrated to 1 ml on the steam bath and the phenol treatment continued as described. Six phenol treatments were usually required to free *Pseudomonas aeruginosa* of nitrogen compounds. Since the residue (presumably carbohydrate in character) was appreciably soluble in the phenol solution, the volumes were kept small.

After the final phenol treatment, the phenol-insoluble material was centrifuged, and most of the phenol washed out with three 50 ml portions of acetone. The precipitate so obtained was dried *in vacuo* over sulfuric acid. The dried material was then mixed with 5 ml of water and heated on the steam bath. After cooling, a few drops of toluene were added, and the solution dialyzed against 1 liter of distilled water overnight. The water was changed three times during the dialysis. The resulting solution was slightly turbid and therefore was clarified by centrifuging for 2 to 4 hours at 5000 R P M in the angle centrifuge. The clear supernatant liquid was stirred well during the slow addition of 10 volumes of acetone. The resulting turbid suspension was coagulated by the addition of 1 to 2 ml of glacial acetic acid. The precipitate was centrifuged off, washed free of acetic acid with acetone, and dried *in vacuo* over sulfuric acid. Yield, 0.2 to 0.5 gm.

The preparations from the typhoid material, *Proteus vulgaris*, and *Pseudomonas aeruginosa* contained no nitrogen by the sodium fusion test. They gave no tests for sugars unless hydrolyzed by dilute acid. Calcium was determined qualitatively in the ash of the products by microscopic examination (11) for the characteristic crystals of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$. Phosphorus was determined by the method of Fiske and Subbarow (12). Dr D H Moore of the College of Physicians and Surgeons, Columbia University, by the ultracentrifuge method found the molecular weight of the

pyrogen obtained from typhoid vaccine to be 62,000, and the material to be homogeneous in character

TABLE I
Chemical Composition of Pyrogens

	Typhoid	<i>Pseudomonas aeruginosa</i>	<i>Proteus vulgaris</i>
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C	39.28	38.75	35.83
H	6.95	6.53	6.06
N	0	0	0
P	0.38	2.38	0.20
Ash	4.43	12.18	8.33

TABLE II
Tests with Typhoid-Paratyphoid Preparation II

Dosage per kilo rabbit	No. of rabbits	Maximum rises in temperature	Average rise in temperature
γ		C	C
0.08	3	0.2, 0.3, 0.5	0.33
0.23	3	0.3, 0.5, 0.7	0.5
0.5	5	0.2, 1.3, 0.4, 0.6, 1.3	0.76
1.0	5	0.9, 0.3, 1.0, 0.3, 0.7	0.64
1.0	5	1.0, 1.2, 0.9, 0.4, 0.4	0.78
1.0	5	0.7, 0.8, 0.7, 1.2, 1.0	0.88
2.0	5	1.2, 1.5, 1.3, 1.4, 1.3	1.34
2.0	5	1.9, 1.5, 1.1, 1.0, 1.2	1.34
2.0	3	1.7, 1.6, 1.6	1.63
2.5	5	1.0, 1.7, 1.2, 1.1, 1.4	1.28
3.0	5	1.7, 1.8, 1.3, 1.5, 2.2	1.70
4.0	4	1.1, 1.3, 1.3, 1.2	1.23
10	3	1.5, 1.6, 1.3	1.47
25	5	2.5, 2.2, 2.2, 2.0, 1.7	2.12
50	5	2.5, 2.6, 2.6, 2.7, 2.8	2.64
200	1	2.3	
300	1	2.4	
400	2	2.3, 2.5	
500	2	1.5 (Died), 2.6	
1000	1	2.6	
2000	4	2.3, 2.2, 1.7 (Died), 1.6	
3000	2	2.6, 1.7	

Typical analyses of the pyrogens obtained from the three different organisms are shown in Table I. For any one organism, preparations from different lots of that organism varied somewhat in composition owing to

variations in the amounts of ash and phosphorus. When calculated to an ash-free basis, the agreement for carbon and hydrogen for that organism was quite close. Atomic ratios for all three pyrogens were close to 1 carbon to 2 hydrogen atoms.

Rabbit Tests of Pyrogen Preparations—The most complete tests were carried out on the nitrogen-free material obtained from the typhoid-paratyphoid vaccines. The results are shown in Table II.

Some irregularities occurred in increased temperatures with increasing quantities of pyrogens, but, in general, the relations are clear. The maximum increase occurred with 25 to 50 γ per kilo of rabbit, the temperature rise being about 2.5°. Larger quantities did not increase this maximum.

TABLE III
Tests with Pseudomonas aeruginosa and Proteus vulgaris

Preparation	Dosage per kilo rabbit	No. of rabbits	Maximum rises in temperature
	γ		°C
<i>Pseudomonas aeruginosa</i> , No. II	0.1	2	0.5, 0.5
	1.0	2	0.3, 0.3
	2.0	2	2.1, 1.2
	5.0	2	2.0, 1.6
	10	5	0.9, 1.0, 1.8, 2.1, 2.2
	300	1	2.2
	500	1	2.6
" " " III	10	6	1.1, 1.8, 1.7, 1.4, 1.7, 0.9
	100	1	2.3
<i>Proteus vulgaris</i> , No. II	0.1	2	0.3, 0.3
	0.5	2	0.7, 0.2
	1.0	2	1.2, 0.6
	2.0	2	0.6, 1.1
	10	1	2.0

Fewer rabbits were used for the higher concentrations, but results of these tests may be grouped. Only occasional deaths occurred. Similar results were obtained with a different preparation. Toxicity tests are not reported in this connection.

Results for the animal tests of pyrogens from the other sources are presented in Table III. These results, although fewer in number, are similar in character to those obtained with the typhoid pyrogen.

DISCUSSION

The results presented in this paper are purely experimental in character. They show that the pyrogens isolated from three different microorganisms when purified did not contain nitrogen. Analyses and tests indicated a

probable carbohydrate character. It is too early to state that they are pure chemical individuals. The rabbit tests showed their active pyrogen properties. In this connection the question may be raised whether the amount of material injected per kilo of rabbit resulting in a maximum temperature rise (approximately 2.5°) would be a satisfactory and useful standard for determining pyrogen activity.

The present study of pyrogens was initiated in the Laboratory of Industrial Hygiene, Inc., in association with the Department of Organic Chemistry of Columbia University. The study is under the direction of Dr. K. George Falk of the Laboratory of Industrial Hygiene, Inc., Professor John M. Nelson of Columbia University, and Professor George B. Wallace of New York University College of Medicine. Only purification of pyrogens and their pyrogenic actions were presented in this paper. The study is being continued. In the work so far, thanks are due Dr. Co. Tui of New York University College of Medicine for his advice and suggestions based on extended experience in this field, Dr. W. G. Malcolm of the Lederle Laboratories, Inc., for supplying much of the initial material used for the preparations, and Dr. D. H. Moore of the College of Physicians and Surgeons of Columbia University for carrying out the molecular weight determinations.

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METABOLISM OF PHOSPHORYLCHOLINE

II PARTITION OF PHOSPHORYLCHOLINE PHOSPHORUS BETWEEN BLOOD PHOSPHATE FRACTIONS III PARTITION OF PHOSPHORYLCHOLINE PHOSPHORUS BETWEEN TISSUES IV DISTRIBUTION OF PHOSPHORYLCHOLINE PHOSPHORUS IN TISSUE LIPIDS*

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In a recent discussion of the application of labeling agents to the study of phospholipid metabolism, Chaikoff (1) has pointed out the various mechanisms by which phospholipid molecules may be assembled. The details of the biosynthetic mechanism are not known but in view of the various bonds whose formation is involved, several intermediates have been suggested (2, 3). The most attractive of these from the experimental standpoint are the glycerol phosphate and nitrogen base phosphate compounds. Several of these possibilities have already been submitted to preliminary examination. Chargaff and Keston (2) have shown the incorporation of the phosphorus of aminoethylphosphoric acid ester in lecithin and cephalin of rat liver and intestine. Taurog¹ (unpublished experiments summarized by Chaikoff (1)) has shown the incorporation *in vitro* and *in vivo* of both glycerophosphate and phosphorylcholine phosphorus in liver and kidney phospholipid, although the incorporation as inorganic phosphate was not precluded in either case.

Independent consideration of the possible mechanisms of phospholipid formation suggested the further investigation of phosphorylcholine as an intermediate in phospholipid metabolism. While this nitrogen base phosphate, the functional unit present in both lecithin and sphingomyelin, has been isolated from normal beef liver by Inukai and Nakahara (4), it is recognized that the appearance of this ester might be attributed to isolation procedures rather than to metabolic processes. Accordingly, an extension of our information on the metabolism of phosphorylcholine was considered of value.

The present report contains data from about 80 experimental and control animals. The experiments were designed to follow the phosphorus from phosphorylcholine into the various blood phosphorus fractions through a

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¹ Taurog, A., personal communication.

directly on a second aliquot by the Tisdall colorimetric adaptation (10) of the Embden-Fetter method

A second aliquot of the original trichloroacetic acid filtrate was taken for determination of the radioactivity of the total acid-soluble phosphorus and a third aliquot for phosphorus analysis. Colorimetric phosphorus analyses on the blood filtrates, made after acid ashing with sulfuric acid, were run in duplicate by the less tedious method of Kuttner and Lichtenstein (12)

The values for specific activity of inorganic and acid-soluble phosphorus fractions of blood calculated for Group II are plotted against time in Fig 1. Results obtained on the rats of Group I, which were handled in similar

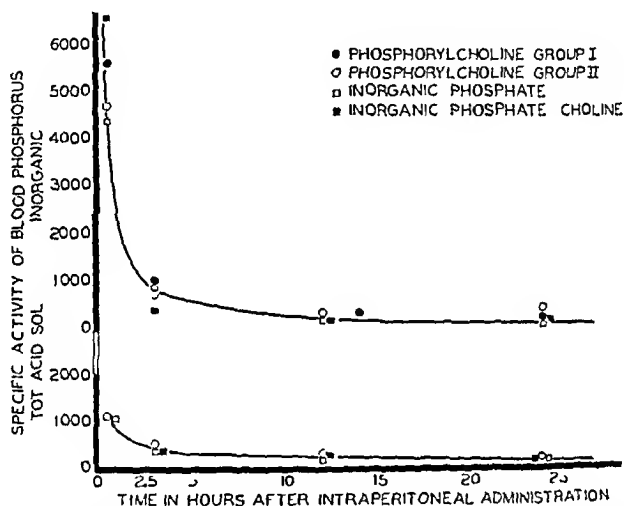


FIG 1 Specific activities of total acid-soluble and of inorganic phosphorus of blood obtained from animals which received phosphorylcholine, phosphate, and phosphate plus choline. Each point represents a single value obtained on the pooled blood of three rats

fashion, are also presented in Fig 1. All animals of Group I received phosphorylcholine but data were not obtained on the total acid-soluble phosphorus, the animals received approximately half the amount of phosphorylcholine received by those in Group II.

Corroborating these data for Groups I and II, additional 12 hour values have been obtained on older animals (Groups III and IV). Data on blood fractions of Group IV are included in Table II, see also whole blood data on Group III rats in Table III. No significant differences in specific activities or percentages of dose fed were detected in the total blood phosphorus, acid-soluble, inorganic, or organic phosphorus of groups receiving phosphorylcholine, phosphate, or phosphate plus choline, respectively.

Specific activity values for inorganic phosphorus in Fig 1 are identical, within the range of experimental error, for all rats, whether they received phosphorylcholine, phosphate, or phosphate plus choline. The same is true for the specific activities of acid-soluble phosphorus. The spread of points about the 30 minute period is probably due to small differences in the period from time of injection to death. The higher value for the group receiving phosphorus plus choline could be attributed to a greater rate of absorption due to splanchnic vasodilatation resulting from parasympathetic stimulation by the choline, an effect less marked with an equivalent amount of phosphorylcholine (13).

Further calculations based on the data presented in Fig 1 from animals killed 30 minutes after injection of phosphorylcholine permit two definite conclusions regarding the metabolic fate of the ester. First, rapid hydroly-

TABLE II
Specific Activities of Blood Phosphorus Fractions

All specific activity figures for Group IV have been corrected for the difference in counts administered to this group (see the fifth column of Table I)

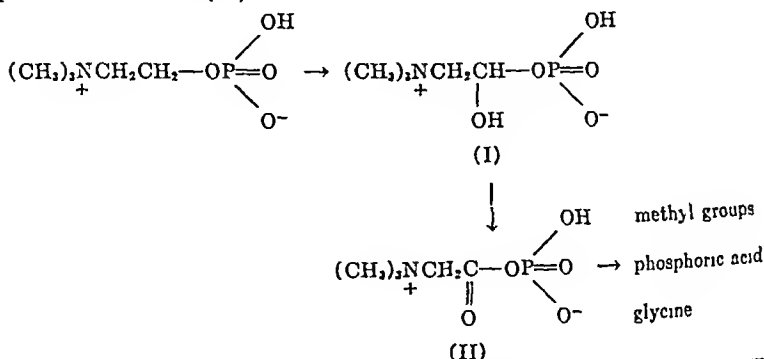
Subgroup (No of rats)	Total P (whole blood)			Total P (acid soluble)		
	mg per cent	specific activity	per cent dose per ml	mg per cent	specific activity	per cent dose per ml
PCh (4)	53	501	0.0220	32	557	0.0150
P (4)	51	485	0.0204	33	634	0.0176
P + Ch (3)	49	481	0.0196	33	610	0.0168
	Inorganic P (acid soluble)			Organic P (acid soluble)		
	mg per cent	specific activity	per cent dose per ml	mg per cent	specific activity	per cent dose per ml
PCh (4)	4.8	578	0.0023	28	554	0.0126
P (4)	5.5	545	0.0025	28	654	0.0152
P + Ch (3)	5.7	541	0.0026	27	630	0.0143

sis of phosphorylcholine to give inorganic phosphate accounts for a large fraction of the administered compound. The high specific activity values for inorganic phosphate in the phosphorylcholine group cannot be accounted for on the basis of the contaminant inorganic phosphate received by these groups (0.2 per cent of the injected count (5)), since calculation shows that the count in the inorganic phosphorus of the group sacrificed 30 minutes after receiving phosphorylcholine was derived from 3.6 per cent of the injected dose. As already pointed out, the ester is not precipitated by the phosphorus reagent, and accordingly a sizable fraction must have undergone hydrolysis.

Second, the hydrolysis of phosphorylcholine is only one of the several mechanisms by which this ester is removed from the circulating blood. From the radioactivity and phosphorus values for total acid-soluble and inorganic phosphate, it has been calculated that in the phosphorylcholine

subgroup after 30 minutes only 1.3 per cent of the radiophosphorus administered was present in organic form in the isolated blood. This must indicate that a maximum of 1.3 per cent of the administered ester remained in the acid-soluble blood fraction after this period. Undoubtedly much less than 1.3 per cent existed as phosphorylcholine, as a similar calculation shows that 1.4 per cent of the inorganic phosphate administered to the control group was present as ester phosphorus in 30 minutes. Accordingly, the ester must disappear rapidly from the circulation, partially through the hydrolysis noted above as well as by diffusion of the intact ester. The metabolic utilization of the ester without liberation of inorganic phosphate would account for a further fraction.

This marked lability *in vivo* of phosphorylcholine is in sharp contrast to the general stability of betaines *in vitro*. The marked stability of phosphorylcholine to acid, base, and oxidizing agents (14) as well as tissue and enzyme preparations (3, 6, 13)² exemplifies the stability of this betaine *in vitro*. In line with our finding of a metabolic lability, phosphorylcholine has been shown to possess lipotropic activity in liver and the ability to furnish labile methyl groups (3). Since sufficient comparable data of a quantitative nature are lacking, the activity of the methyl donor may be attributed to choline freed by hydrolysis from the ester. The interesting possibility of an oxidatively coupled transfer of methyl groups from phosphorylcholine is, however, consistent with the present limited information on the mechanism of phosphorylcholine metabolism and methyl transfer from choline. Thus, stepwise oxidation of phosphorylcholine through betaine aldehyde phosphate (I) to betaine phosphate (II) would evolve an energy-rich phosphate, which by cleavage on the proper enzyme substrate might provide the energy source for methyl transfer. Glycine, which would be formed through such a mechanism, is probably the normal demethylation product of choline (15).



² Lan, T. H., and Riley, R. F., unpublished observations

A complete description of the rate of entry of phosphorylcholine into circulating blood and evaluation of the rate of hydrolysis to inorganic phosphate, following intraperitoneal injection, will require further data obtained for intervals of time less than 30 minutes. From a casual inspection of Fig 1, the hypothesis might easily be formed that the ester is rapidly and completely split *in vivo*. However, the data presented in Fig 1, on the disappearance of blood inorganic phosphate derived from phosphorylcholine, may be logically interpreted without assuming total hydrolysis of the ester. Rapid removal of the greater portion of the circulating phosphorylcholine by an organ capable of rapid hydrolysis of the ester would result in initial maintenance of blood inorganic phosphate of high activity without requiring the total hydrolysis of the ester. Thus, not more than 4 per cent of the injected phosphorylcholine phosphorus was required to provide the count in the blood inorganic phosphate at 30 minutes.

III Partition of Phosphorylcholine Phosphorus between Tissues

If phosphorylcholine were not completely hydrolyzed, and promptly, certain organs might be expected to pick up the unhydrolyzed ester preferentially. Therefore, if organs are found in which phosphorylcholine is picked up preferentially as compared with the amount of inorganic phosphate taken up in comparable experiments, it would provide evidence for the supposition of incomplete hydrolysis. To delineate such tissues, phosphorus distribution was studied on whole tissue samples of older animals of Groups III and IV administered phosphorylcholine, phosphate, and phosphate plus choline.

The animals of Groups III and IV were divided into subgroups, injected as indicated in Table I, and placed in individual metabolism cages arranged for collection of urine and feces. 12 hours after injection the animals were killed by exsanguination. The whole blood from the individual rats of Group III was measured volumetrically and analyzed without fractionation. The blood of animals in Group IV was handled as described for Groups I and II except that the blood was aspirated into sodium oxalate tubes prior to trichloroacetic acid precipitation in order that an aliquot of whole blood could be obtained for total phosphorus determination. The other tissues were removed promptly and weighed into 50 ml beakers or 200 × 25 mm test-tubes for ashing. Usually the whole tissues of each animal were ashed repeatedly with fuming nitric acid to give homogenous solutions which were measured for radioactivity. Two exceptions to this procedure were that the eyes from Group IV were pooled by subgroups to provide sufficient material, and that only a lobe of the livers of Group III was ashed, the remainder being saved for the lipid analyses described in Section IV.

All analyses of tissue phosphorus, carried out in duplicate, were made on aliquots of the homogenous solutions used for radioactivity measurement.

A second treatment with sulfuric acid and superoxol was required to oxidize water-soluble organic material remaining in the partially ashed solutions, and to eliminate nitric acid which interferes in the phosphorus determination of Kuttner and Lichtenstein. Summaries of the percentages of the administered radioactivity found per gm of various tissues and specific activities and phosphorus percentages for whole tissues calculated from phosphorus analyses and radioactivity measurements are presented in Fig 2 and Table III, respectively.

From the data presented in Fig 2 and Table III, it may be noted that differences of large order are not discernible between phosphorylcholine, phosphate, and phosphate plus choline subgroups, with the exception of the livers of Group III (Table III). The differences in phosphorus percentages

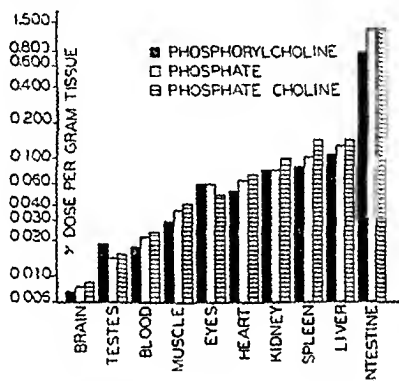


FIG 2 The percentages of administered radioactivity found per gm of various fresh tissues. The values for testes and eyes are from Group IV, all others are from Group III. A logarithmic scale has been employed on the ordinate.

found here between subgroups consistent in themselves we are inclined to attribute to faulty adjustment in pH of the solutions on which the phosphorus determinations were carried out. Such an explanation is quite possible, since liver samples for each subgroup were analyzed together, those of the subgroups at different times. Since specific activities depend on these values, they too are of questionable significance.

The tissues of animals which received phosphorylcholine may show a significant tendency toward lower values (percentage dose per gm of tissue) as compared to phosphate controls (Fig 2), since retention of phosphate is definitely indicated by the excretion data summarized in Table IV. Application of Fisher's *t* test (16) to these latter values indicates a high order of significance ($P < 0.01$) of the differences between phosphorylcholine and phosphate and between phosphorylcholine and phosphate plus choline. This finding is taken as evidence that phosphorylcholine is not completely

TABLE III

Specific Activities and Phosphorus Percentages for Whole Tissues of Rats of Groups III and IV

It should be noted that specific activities reported for Group IV in this table are not directly comparable to the specific activities for Group III, but are only proportional, since Group IV received a greater number of counts per gm of rat than Groups I, II, and III (see Table I)

Tissue	Subgroup	Average P (range)	Specific activity (range)	Tissue	Subgroup	Average P (range)	Specific activity (range)
Group III							
Liver	PCh	<i>per cent</i> 0 29 (0 27-0 32)	457 (354-570)	Muscle	PCh	<i>per cent</i> 0 28 (0 25 -0 32)	133 (104 -150)
	P	0 48 (0 45-0 51)	266 (233-302)		P	0 26 (0 22 -0 31)	140 (96 -190)
	P + Ch	0 37 (0 31-0 37)	460 (421-490)		P + Ch	0 24 (0 23 -0 28)	179 (155 -226)
Intes- tine	PCh	0 28 (0 26-0 33)	354 (263-400)	Heart	PCh	0 24 (0 22 -0 27)	314 (186 -403)
	P	0 29 (0 25-0 34)	416 (355-488)		P	0 22 (0 19 -0 27)	295 (267 -328)
	P + Ch	0 31 (0 30-0 32)	452 (439-466)		P + Ch	0 21 (0 20 -0 22)	399 (391 -411)
Kidney	PCh	0 32 (0 28-0 34)	319 (231-402)	Brain	PCh	0 31 (0 27 -0 35)	30 2 (20 2- 36 6)
	P	0 30 (0 27-0 34)	270 (223-321)		P	0 33 (0 32 -0 34)	24 1 (19 5 -28 6)
	P + Ch	0 31 (0 28-0 33)	356 (333-379)		P + Ch	0 32 (0 30 -0 33)	28 6 (25 3- 32 5)
Spleen	PCh	0 38 (0 31-0 42)	282 (175-458)	Blood	PCh	0 046 (0 041-0 050)	520 (447 -580)
	P	0 35 (0 29-0 40)	301 (227-382)		P	0 047 (0 044-0 053)	438 (332 -527)
	P + Ch	0 34 (0 31-0 37)	449 (386-513)		P + Ch	0 045 (0 039-0 050)	574 (548 -590)
Group IV							
Testes	PCh	0 22 (0 21-0 25)	135 (114-159)	Eyes	PCh	0 12	126
	P	0 22 (0 21-0 23)	104 (93-115)		P	0 12	136
	P + Ch	0 22 (0 20-0 24)	108 (93-121)		P + Ch	0 12	138

hydrolyzed in a brief period. Further work is in progress in an attempt to account for the indicated retention of phosphorylcholine phosphorus.

No large differences in specific activities of whole tissue phosphorus were found among the major organs of rats whether they received phosphorylcholine or inorganic phosphate. Conceivably, however, large differences might exist in specific phosphorus fractions of these tissues, and compounds in which phosphorylcholine exists as a moiety, *i.e.* lecithin and sphingomyelin, would be of special interest. As a matter of fact, the possibility that phosphorylcholine is an intermediate in lecithin and sphingomyelin synthesis and that this important hypothesis could be tested by use of phosphorylcholine with radiophosphorus was the point of departure for this entire study. Preliminary investigation of this possibility has been made. These results, obtained on animals used for studies of the partition of phosphorus in the blood and general tissue distribution are presented in the following section.

TABLE IV
Urinary and Fecal Excretion of P^{32} 12 Hours after Administration

Subgroup	No. of rats	Combined urinary and fecal excretion	
		Average	Range
		<i>per cent of total dose</i>	<i>per cent of total dose</i>
PCh	12	12.9	9.1-18.2
P	12	17.4	12.5-23.7
P + Ch	9	17.7	14.4-23.2

IV Distribution of Phosphorylcholine Phosphorus in Tissue Lipids

The treatment of the animals of Groups III and IV employed in these experiments has been described above. Tissues taken from these animals for isolation of lipid fractions were removed immediately after sacrifice, weighed, and ground with sand. The finely ground tissues were repeatedly extracted with alcohol-ether and then with chloroform-methanol, the filtered solvent extracts were worked up for phospholipid by the usual procedures (17-19). With the exception of the sphingomyelin samples noted later, all phospholipid samples were completely ashed with concentrated sulfuric acid prior to counting and diluted to a known volume. Aliquots of this solution were then taken for measurement of radioactivity and duplicate phosphorus analyses.

Results and discussion of the lipid experiments are presented in two sections which deal, respectively, with the total acetone-precipitable phospholipid and with lecithin, cephalin, and sphingomyelin fractions obtained from plasmalogen-free phospholipid.

Total Phospholipid—Preliminary data for lipid phosphorus were obtained from the livers of Group III. The data, summarized in Table V, are for total phospholipid.

The specific activities and percentage doses in Table V show that the animals which received phosphorylcholine evidence a decreased uptake of radiophosphorus in liver phospholipid after 12 hours as compared with those animals which received inorganic phosphate

If total hydrolysis of the ester had occurred in a brief period, data from phosphorylcholine and phosphate plus choline subgroups should be nearly identical after an extended interval. Partial hydrolysis, by liberating inorganic phosphate, would tend to obscure data indicative of utilization of the ester in phospholipid synthesis. Since a decreased uptake of radiophosphorus by liver phospholipid was found after 12 hours, we must suppose phosphorylcholine has produced a decreased rate of turnover of phospholipid phosphorus or an enhanced turnover with a consequent fall 12 hours after injection. On the basis of evidence obtained on liver lecithin, cephalin, and sphingomyelin fractions at 12 hours, we are inclined to attribute the

TABLE V

Specific Activities and Percentages of Dose per Gm in Total Liver Phospholipids 12 Hours after Administration of P³²

Subgroup	No. of values averaged	Liver phospholipid (range)	Per cent dose per gm phospholipid (range)
		<i>specific activity</i>	
PCh	8	398 (318-444)	1.33 (1.06-1.49)
P	6	582 (495-630)	2.20 (1.88-2.44)
P + Ch	5	575 (558-597)	1.97 (1.90-2.05)

12 hour differences noted in Table V to a decreased rate of turnover of liver phospholipid phosphorus. Further work is required to decide this question.

Lecithin, Cephalin, and Sphingomyelin Fractions—The total phospholipid obtained from livers and kidneys of animals in Group IV by the precipitation of crude lipid extracts with acetone was treated to remove plasmalogens by the method of Neuman (20). The plasmalogen-free phospholipids so obtained were dissolved in 2 to 3 ml of warm ethyl alcohol. After 12 hours refrigeration, the lecithin and cephalin fractions obtained in the liquid and solid phases, respectively, were made up separately in volumetric flasks without further reprecipitation. Ashed aliquots of the crude lecithin and cephalin fractions were counted, and further aliquots employed for the determination of phosphorus and choline, and also for total nitrogen when sufficient material was available. Choline was determined colorimetrically as the renechate, nitrogen by the usual semimicro-Kjeldahl procedure.

No attempt was made to obtain quantitative separations of preparations

recognized as containing complex mixtures of lecithins and cephalins, however, the percentages of choline phosphatides in both lecithin and cephalin fractions have been determined. From radioactivity measurements of each "lecithin" and "cephalin" fraction and the percentages of choline phosphatide in each, the specific activities of "lecithin" phosphorus (phosphorus of choline phosphatides) and the specific activities of "cephalin" phosphorus (phosphorus of choline-free phosphatides) have been calculated for each subgroup. The similarity of N/P ratios for lecithin and cephalin fractions of each subgroup (Table VI) indicates that this calculation has been made for comparable fractions. Such a procedure possesses an advantage in that

TABLE VI

Composition of Lecithin and Cephalin Fractions Obtained from Livers and Kidneys of Rats of Group IV 12 Hours after Injection of P³²

Tissue	Subgroup	Lipid fraction	Lecithin*	N/P ratio
			<i>per cent</i>	
Liver	PCh	Lecithin	72	1.08
	P	"	71	1.11
	P + Ch	"	78	1.12
"	PCh	Cephalin	40	0.81
	P	"	29	0.90
	P + Ch	"	52	0.88
Kidney	PCh	Lecithin	60	1.17
	P	"	58	1.16
	P + Ch	"	56	1.17
"	PCh	Cephalin	26	
	P	"	25	
	P + Ch	"	22	

$$* \text{ Per cent lecithin} = \frac{\text{mg choline} \times 6.58 \times 100}{\text{mg P} \times 25}$$

values obtained do not reflect differences due to an exhaustive reprecipitation with loss of the more soluble lecithin and cephalin fractions.

When sufficient material remained, the lecithin and cephalin fractions were recombined and worked up for sphingomyelin. This was carried out by evaporating the combined extracts to dryness and dissolving the residue in absolute methanol. The sphingomyelin was precipitated from the methanol solution by the reineckate method as modified by Hunter (19). The lungs of the animals in Group IV were extracted as previously described and sphingomyelin isolated as the reineckate from the total lipid extract. Reineckate preparations were counted directly by solution in methanol dioxane, 1:1. Aliquots of the counted sample were then ashed with sulfuric acid for determination of phosphorus. These data are summarized in Tables VI and VII.

From Table VII, it may be seen that phosphorylcholine phosphorus enters lecithin, cephalin, and sphingomyelin fractions. This was anticipated in view of the rapid appearance of the phosphorus of phosphorylcholine in the inorganic phosphate of blood.

As pointed out above, partial hydrolysis of phosphorylcholine to inorganic phosphate would tend to obscure differences due to utilization of phosphorylcholine as contrasted with inorganic phosphate or phosphate plus choline, in lecithin, cephalin, and sphingomyelin synthesis. However, differences might be expected in these fractions, since differences between subgroups were noted in total phospholipid in the previous experiment. If phosphorylcholine is presumed to be an immediate precursor of lecithin or sphingomyelin or of both, these fractions should show higher specific activities for subgroups receiving phosphorylcholine than those from animals which received inorganic phosphate. Actually no differences were found.

TABLE VII

Specific Activities of Lecithin, Cephalin, and Sphingomyelin of Rat Livers, Kidneys, and Lungs 12 Hours after Injection of P^{32}

Tissue	Subgroup	Lecithin P	Cephalin P	Sphingomyelin P
Liver	PCh	966	363	648
	P	825	735	619
	P + Ch	921	495	593
Kidney	PCh	649	375	
	P	590	344	
	P + Ch	551	357	
Lung	PCh			382
	P			371
	P + Ch			364

The differences, contrary to expectation, appeared in the cephalin or non-choline phospholipid fraction rather than in the lecithin and sphingomyelin fractions, and here the subgroup receiving phosphorylcholine (liver) shows a lower specific activity than the groups which received inorganic phosphate. This would account for the decreased uptake of total phospholipid phosphorus found in the first experiment. It is interesting that this depressed uptake is not shown in the kidney cephalin fraction from the phosphorylcholine subgroup.

Chargaff and Keston (2), in tracing the utilization of aminoethylphosphoric acid containing radiophosphorus in the synthesis of body phosphatides, have found that the phosphorus of this ester likewise appears rapidly in lecithin and cephalin of both liver and intestine of the rat. In their experiments the uptake of the phosphorus of aminoethylphosphoric acid ester in liver cephalin was relatively slower than in liver lecithin as com-

pared to their inorganic phosphate controls (21). If the depressed uptake evidenced in experiments with aminoethylphosphoric acid and phosphorylcholine is ascribed to the inability of the organism to utilize the intact esters in lieu of inorganic phosphate, the decreased uptake should be apparent in all phospholipid fractions.

The depression of the uptake of phospholipid phosphorus by either aminoethylphosphoric acid ester or phosphorylcholine could be ascribed to a blocking of the enzyme systems involved in the esterification by these esters which have space-charge distributions similar to those present in ethanolamine cephalin or in lecithin. However, the inhibition should be particularly pronounced in the lecithin fraction rather than in the cephalin in the case of phosphorylcholine. Studies on the rate of uptake of phosphorylcholine phosphorus by these fractions are indicated as the next step in this work, and may provide an explanation for the anomalous effect on the uptake of cephalin phosphorus. On the basis of present evidence, it appears unlikely that phosphorylcholine functions directly, if at all, in the biosynthesis of lecithin or sphingomyelin.

In the light of recent evidence (20), it would appear more probable that cephalin and perhaps lecithin and sphingomyelin are not formed directly from their constituent parts but rather evolve from more labile complex intermediates which are in rapid equilibrium with simple precursors. Because of initial, rapidly equilibrating processes, it is quite possible that the differences in specific activities of radiophosphorus of the isolated phospholipid fractions might be small, following the injection of simple precursors containing radiophosphorus or radioactive phosphate itself. This possibility has not been ruled out in the case of phosphorylcholine, indeed, the rapid appearance of the phosphorus of phosphorylcholine in the inorganic phosphate of blood could be interpreted as indicative of such a process. Further work on this problem is in progress.

SUMMARY

Phosphorylcholine phosphorus appears rapidly as inorganic phosphorus in the circulating blood of the rat following intraperitoneal injection of the ester. Simultaneously, the ester disappears rapidly from the circulation. This lability *in vivo* is in marked contrast to the stability exhibited by this betaine *in vitro*.

Phosphorylcholine phosphorus is excreted somewhat more slowly than inorganic phosphate, however, the phosphorus of phosphorylcholine shows only small, if significant, differences in whole tissue distribution as compared to inorganic phosphorus in brain, testes, blood, muscle, eyes, heart, kidney, spleen, liver, and intestine.

Preliminary evidence indicates that phosphorylcholine inhibits the turn

over of total phospholipid in the liver. The inhibition appears limited to the non-choline phosphatide fraction rather than to the expected lecithin fraction. No evidence was adduced to show that phosphorylcholine as a unit is utilized in the synthesis of phospholipids.

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THE FATE OF METHYLCELLULOSE IN THE HUMAN DIGESTIVE TRACT

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Methylcellulose has recently been employed as a thickening agent in the pharmaceutical and food industries. Although it is essentially innocuous for animals when administered by various routes (1, 2), it appeared desirable to measure the extent to which the material can be recovered from the feces after administration of single doses to human subjects on various diets.

Grateful acknowledgment is made to The Dow Chemical Company for the financial sponsorship of the investigation and for the supply of the methylcellulose used.

The commercial product consists of a partially depolymerized cellulose¹ in which on a statistical average two methyl radicals are held in ether linkage in each glucose unit (methoxyl content, 30.49 per cent, theory for two methoxyl groups per glucose unit, 32.63 per cent). In view of the possibility of the metabolic rupture of the ether linkage (3), an effort was also made to determine whether absorption of methanol or its oxidation products occurs after the ingestion of the product.

Two male adults and one 10 year-old girl were employed as subjects in ten experiments. The collaboration of the medical and nursing staffs of the Children's Convalescent Home in Cincinnati, Ohio, in the study of the latter subject is gratefully acknowledged. In each experiment, 10 gm of methylcellulose were given in a single dose except in Experiment 9, in which 5 gm were given. The product was taken as a 5 per cent solution, except in one experiment in which it was eaten as a gel. Following each dose, all fecal evacuations for from 1 to 4 weeks were dried individually and their content of methoxyl groups determined. Control samples of feces were found to contain variable amounts of substances titrating as methoxyl groups. This made it necessary to isolate the methylcellulose. Since the latter is soluble in cold water but is coagulated by hot water, the dried samples were treated with boiling water to coagulate the methylcellulose and to remove the soluble interfering substances. After filtration, the methylcellulose was extracted from the residues by prolonged treatment with cold water, giving filtrates suitable for Zeisel determina-

¹ Commercial methocel is available in a variety of types with respect to viscosity and molecular weights, the latter ranging from 30,000 to 150,000.

tions In a representative test of the method, in which 6.9 gm of methyl cellulose were added to 22.6 gm of dried feces, 99.8 per cent was recovered

In the last six experiments, the methoxyl groups extracted during the preliminary treatment with hot water, as well as those remaining in the residues after the cold water extraction of methylcellulose, were also determined These values were then compared with the mean output of methoxyl groups in the corresponding fecal fractions obtained during control periods in which the subjects were on similar diets

Hydrolysis of the methyl ester groups of pectin gives rise to methanol After a day during which he ate nothing but twenty apples, von Fellenberg (4) recovered from his urine 8.7 mg of methanol, or 0.9 per cent of the amount liberated from twenty similar apples by mild alkaline hydrolysis In three of our experiments, the urine was studied for evidence of the absorption of methanol or its oxidation products Since methanol is but slowly oxidized in the body, it may be detected in the urine for 3 or 4 days following a small dose (5) Although the intermediary oxidation product, formaldehyde (6), has not been detected in the urine of animals following the administration of small quantities, an increased excretion of formic acid has been reported (7) More recently, Voit (8) stated that the use of a diet high in pectins increases the 24 hour urinary excretion of formic acid to 30 to 45 mg, the usual excretion on a mixed diet being only 6 to 14 mg Werch and Ivy (9), however, were unable to confirm this observation

Methods

Methylcellulose—The entire sample of feces was weighed, dried in an oven at 105°, and reweighed It was then boiled for half an hour with 10 ml of water per gm of dried feces to coagulate the methylcellulose After the solution was filtered through a mushin cloth, the filtrate was concentrated to about 100 ml, boiled, and again passed through the cloth The cloth together with the coagulated methylcellulose and fecal residues was covered with about 60 ml of water per gm of dried feces, and allowed to stand overnight in a refrigerator The liquid was then filtered through the cloth, the extraction procedure repeated with 40 ml of cold water per gm of dried feces, and the filtrates united (In the case of samples containing large amounts of methylcellulose, the cold water extraction was again repeated, the third filtrate was analyzed separately) Small aliquot portions (5 to 10 ml) of the filtrates were evaporated to dryness in a Zeiss vessel, by the aid of a current of air or carbon dioxide, following which the methoxyl content was determined by a modified volumetric procedure as employed in the laboratories of The Dow Chemical Company (10)

Since it appeared possible that traces of interfering compounds not removed by the hot water treatment might contaminate the cold water

extract of methylcellulose, the magnitude of any error due to this cause was determined by applying the method to twenty-three samples of feces obtained from a subject on a normal diet. 300 ml of boiling water were used for the first extraction. The mean methoxyl content was 0.013 ± 0.0006 gm (standard deviation ± 0.0049 gm, coefficient of variability 38.02 per cent), corresponding to about 0.14 ml of thiosulfate solution consumed in the analyses. This amount was deducted from the volume of thiosulfate used in all recovery experiments but the first two, in these, a somewhat greater correction (0.2 ml of thiosulfate solution) was made and as a result these recoveries may have been slightly low.

Formic acid was determined in the urine by the method of de Eds (11), modified by collecting only the first liter of steam distillate and by beginning its evaporation before all portions had been collected. Control analyses in which 1 mg of formic acid was added to water gave a recovery of 99.8 per cent.

Methanol was determined by the method of Harger (12), but the urine was made alkaline before distillation to avoid contamination of the distillate by formic acid.

Results

Recovery of Unaltered Methylcellulose—Fig. 1 shows the cumulative recovery of unaltered methylcellulose in the cold water extracts of specimens of feces obtained during the first 4 days following single 10 gm doses given to two adult male subjects (designated Subjects A and B in Table I) in the first eight experiments. A 10 year-old girl ingesting a diet low in pectans was Subject C of the last two experiments, 5 gm of methylcellulose having been given in Experiment 9, and 9.8 gm in Experiment 10.

In the last six experiments the amounts of methoxyl groups derived from ingested methylcellulose and present in the hot water extracts and final residues were obtained by deducting from the results of the daily analyses of these fractions the mean daily excretion of interfering substances in each. The latter amounts were previously determined by daily analyses of these fractions of the feces of each subject collected during adequate control periods on the various dietary regimens. The data employed for these corrections are presented in Table I, along with statistical measures of their variability. In Fig. 1, the recoveries of the methoxyl groups ingested as methylcellulose from the hot water filtrates, the recoveries in the final residues, and the total recovery of methoxyl ingested as methylcellulose are represented.

Excretion of Formic Acid and Methanol—During a period of several weeks in which daily determinations were made on the first morning specimens of the urine of Subject C (maintained on a diet low in pectin), no formic acid

was found on 17 days, and concentrations approximating 5 mg per liter were found on 3 days. On three occasions considerably larger amounts were encountered. On 1 day the concentration was 49 mg per liter. At one period, on 3 successive days, values of 12, 20, and 0 mg per liter were found. At another period, the concentrations were 9.6, 10.4, and 45 mg per liter and an undeterminable trace on 4 successive days. In contrast to these occasional sporadic periods of increased excretion of formic acid, no formic acid appeared in the urine on the morning following the administration of 5 gm of methylcellulose at bedtime. A specimen voided at

TABLE I

Amounts of Substances Titrating As Methoxyl in Hot Water Extracts and in Residues of Feces from Subjects on Various Diets

Diet	Normal + 50 gm bran	Sippy	Normal	Sippy	Low pectin
Subject	A	A	B	B	C
No. of days on which samples were obtained	24	4	24	10	21
Mean quantity of methoxyl groups in hot filtrates per sample for 1 day, gm	0.057	0.0288*	0.033	0.0204	0.0107
Probable error, gm	±0.00335	*	±0.0018	±0.0015	±0.0006
Standard deviation, gm	±0.024	*	±0.014	±0.007	±0.004
Coefficient of variability, %	42.34	*	43.12	33.61	37.84
Mean quantity of methoxyl groups in residue per sample for 1 day, gm	0.357†	0.0727*	0.0896	0.046	0.046
Probable error, gm	±0.027	*	±0.0049	±0.0027	±0.0024
Standard deviation, gm	±0.189	*	±0.036	±0.013	±0.019
Coefficient of variability, %	53.03	*	40.16	27.61	40.63

* Average only, too few samples studied for statistical treatment

† Value calculated from twenty-three samples because of loss of one sample during analysis

noon contained 8 mg per liter, but none was found in the urine of the next morning. No more than 3.75 mg of formic acid was excreted by this route during the first 16 hours after the methylcellulose had been taken. Even less, 1.85 mg, was recovered after the ingestion of 9.8 gm of methylcellulose.

Concentrations of 0.29, 1.43, 0.0, 2.66, and 4.8 mg of formic acid per liter were found in five morning specimens of the urine of Subject A, collected while on his customary diet. After he had taken 10 gm of methylcellulose at 10 p.m., the first specimen on the following morning contained 2.00 mg per liter, and others voided 3 and 17 hours after the ingestion of

the methylcellulose had 2.25 and 8.00 mg per liter, respectively. Further specimens during this day were not analyzed, but on the following morning the first specimen had only 2.00 mg per liter. The slight rise found at the 17th hour appears of little significance, since the total amount of formic acid in this and all preceding volumes of urine amounted to only 4.47 mg. This is less than 0.1 per cent of the amount of formic acid expected (4.52 gm), were all of the methoxyl of the methylcellulose liberated, absorbed, and oxidized.

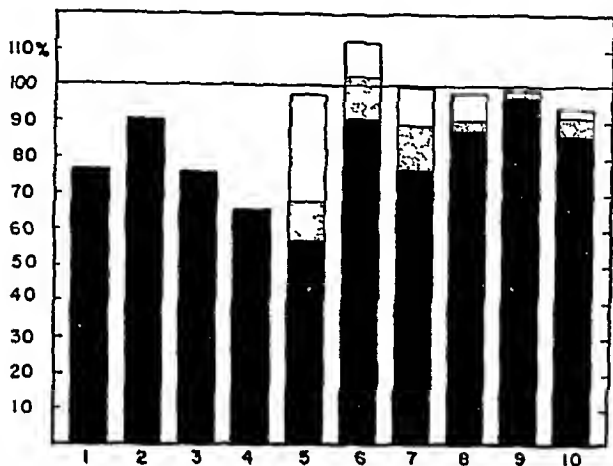


FIG. 1. Accumulated recovery of methoxyl groups in the feces collected during the 4 days following the ingestion of 10 gm of methylcellulose. The solid portions of the bars represent cold water filtrates, the stippled portions, hot water filtrates, the clear portions, the residues. Experiment 1, Subject A, on his customary diet, Experiment 2, milk diet, Experiment 3, customary diet + 10 gm of bran daily, Experiment 4, customary diet + 50 gm of bran daily, Experiment 5, repetition of Experiment 4 after 50 gm of bran daily for a month, Experiment 6, after having returned to a milk diet. Experiment 7, Subject B, customary diet, Experiment 8, milk diet. Experiments 9 and 10, Subject C, low pectin diet (in Experiment 9 only 5 gm of methylcellulose were given).

Daily determinations of the urinary methanol excretion were also made during the study of Subject C. In general, a rise in methanol accompanied each sporadic increase in urinary formic acid excretion, increases also occurred, however, on some days when no increased excretion of the latter could be detected. Neither the 5 gm nor the 9.8 gm dose of methylcellulose was followed by a urinary concentration of methanol exceeding values occasionally encountered during the control periods.

DISCUSSION

Recovery Data—Daily analyses of the feces made over periods of 2 to 4 weeks following each dose in the first four experiments showed that most of the methylcellulose was eliminated within the first 2 or 3 days, and that none could be found after the 4th day.

Comparison of the cumulative amounts recovered at this time (Fig. 1) shows that the inclusion of roughage in the diet tends to prevent the recovery of unaltered methylcellulose. When on a milk diet, started 3 days before taking the methylcellulose (Experiment 2), Subject A had eliminated 90.4 per cent of the ingested methylcellulose by the 4th day, but when he was on his usual diet (Experiment 1) only 76.6 per cent had appeared within the corresponding time. The daily addition of 10 gm of Kellogg's All-Bran (Experiment 3) had no marked effect, 75.8 per cent being eliminated in 3 days, but an increase to 50 gm of bran daily (Experiment 4) decreased the recovery to 65.6 per cent. After the diet high in roughage had been continued for about a month, a repetition (Experiment 5) gave a recovery of but 56.6 per cent in the following 3 day period. Experiment 6, begun 4 days after a milk diet had again been substituted, resulted in the recovery of 90.8 per cent in the cold water extract, a proportion identical with that in Experiment 2 in which the same subject consumed the same diet.

The results were similar in the case of Subject B. A recovery of 76.8 per cent was obtained when the subject adhered to his usual diet (Experiment 7), while being increased to 87.7 per cent (Experiment 8) when he took a milk diet.

The results of Experiments 5 to 10 indicate that the methoxyl groups of ingested methylcellulose not found in the cold water extracts were eliminated in the hot water filtrates and in the final residues. The amount recovered in all three fractions in Experiments 5, 7, 8, and 9 were within 3 per cent of the amounts ingested. The apparently excessive recovery in Experiment 6 is due to an undercorrection for the daily dietary content of substances that reacted like methylcellulose. This occurred because insufficient data on the excretion of interfering substances were available to yield a reliable mean for use in making this correction, only four samples having been analyzed (Table I).

The necessary corrections for interfering substances usually amounted to from 2 to 4 per cent of the amounts of the methoxyl groups given as methylcellulose, and were as great as 13 per cent when a high roughage diet was used. The magnitudes of these corrections and the variability of the data from which they were computed lessen somewhat the accuracy of the results on recoveries in the hot water filtrates and final residues. The

recovery of methoxyl groups in these fractions within any period of 3 or 4 days is subject to error, therefore, in that the output of interfering substances during this period may have deviated from the mean output determined over a much longer control period. However, continuation of the daily analyses in Experiments 5 and 8 for as long as 7 days did not alter the results materially. This source of error does not affect the recovery of methylcellulose from the cold water filtrates, for which no significant corrections for interfering substances are required.

In Experiment 10, considerable persuasion was needed to induce the child to drink the rather large volumes of dilute solution required in order to give 9.8 gm of methylcellulose. The volume was divided into several portions, and it is possible that through failure to rinse the glass after each portion was drunk the entire amount may not have been ingested. For this reason, as well as because of the difficulty attending the accurate collection of the excreta of an immature subject, the slightly lower recovery in this experiment is probably of little significance.

Data regarding the normal occurrence in feces of substances titratable by the Zeisel method reported in Table I do not appear to have been recorded hitherto. The hot water extracts of the feces of Subject B, when on a milk diet, contained on the average 20 mg of such materials. The residues contained 2.2 times as much. Data for Subject A on this diet were fewer in number but indicated a similar distribution between the two fractions. On a freely chosen diet, the amounts excreted by Subject B were greatly increased but the ratio between the amounts in the insoluble and soluble fractions was increased only slightly. This ratio was higher (4.3) in the feces of the child receiving a diet low in pectin. The daily addition of 50 gm of bran to the diet of Subject A doubled the output in the hot filtrates over what it had been when the diet consisted of milk, and more than quadrupled the output in the residues.

Analysis of the bran by the procedure used for the analysis of feces indicated that the daily intake of 50 gm contributed 0.158 gm of water-soluble methoxyl groups, an amount greater than the increment of 0.028 gm found in the hot water filtrates as a result of the feeding of bran. It is apparent, therefore, that much of the water-soluble methoxyl of the bran escaped recovery. The soluble portion of bran consists in part of various pectins. These contain varying amounts of methyl esters which are readily hydrolyzed to methanol (8) and so are rendered available for absorption.

Effects of Diets upon Methylcellulose in Digestive Tract—The appearance of increased amounts of methoxyl groups in the hot water extracts following the administration of methylcellulose is probably to be interpreted as an indication that the macro molecules have been broken into smaller units no longer coagulable by heat. When the diet was low in roughage, only a

small percentage of the ingested dose appeared in this fraction, 28 per cent for Subject B (Experiment 8), and 5.5 per cent for Subject C (Experiment 10). With the inclusion of roughage, this was increased to 11 to 12 per cent (Experiments 5 and 7). The large amount in Experiment 6 is probably in error because of the paucity of the data employed in the necessary correction.

The percentage of the methoxyl groups ingested as methylcellulose which appeared in the fecal residues was markedly increased by the inclusion of roughage. It amounted to 8 to 10 per cent in the case of adults on a milk diet, 11 per cent on a normal diet, and 30 per cent on a high roughage diet. The following experiment indicates that the latter high figure was not due solely to physical interference with the extraction of these residues caused by the presence of bran. 50 gm of bran were allowed to remain for 24 hours in a solution of 5 gm of methylcellulose in 400 ml of water. After the material was dried, 97.6 per cent of the methylcellulose was recovered from the water-soluble fraction. A similar mixture was inoculated with a small amount of fresh feces. After standing for 2 days at room temperature, it was dried and then separated into the usual fractions. From the cold water extracts only 67.7 per cent of the methylcellulose could be recovered. The hot water filtrate yielded 0.152 gm or very nearly the amount extractable from the bran alone (0.158 gm). After correction for the contribution made by the insoluble portion of the bran, the final residues contained 29.5 per cent of the methoxyl groups of the methylcellulose. The total recovered from all fractions was about 96.8 per cent. That the distribution in this experiment was somewhat similar to that in Experiment 5 suggests that under certain circumstances bacteria may exert an action upon methylcellulose.

Excretion of Formic Acid and Methanol.—The conclusion drawn from ten recovery experiments, that little loss of methoxyl occurs during the passage of methylcellulose through the digestive tract, irrespective of the character of the diet consumed, found further confirmation in our failure to obtain conclusive evidence of the excretion of formic acid or of methanol. Slight increases in the urinary concentrations of these substances did occur after the ingestion of methylcellulose, but were much less than those which occurred sporadically at times when no methylcellulose had been taken. Values reported as methanol may also have included other undefined reducing substances, since the method employed depended upon the oxidation of methanol in the distillate from the urine by potassium dichromate.

Several attempts were made to detect an increase in the urinary excretion of formic acid or methanol following the ingestion of large doses of fruit pectin, but only in exceptional instances were positive results obtained. This is in agreement with the observations of Werch and Ivy (9).

SUMMARY

1 In the human subject on normal or low roughage diets, methylcellulose passes through the digestive tract in large part unaltered

2 Increasing amounts of roughage in the diet tend to convert increasing proportions of methylcellulose into products of altered solubility and heat coagulability

3 Irrespective of the accompanying diet, practically all of the methoxyl groups of ingested methylcellulose can be recovered from the feces

4 After the ingestion of large doses of methylcellulose, the amounts of methanol, if any, which are absorbed and excreted as such or after oxidation to formic acid are less than those occasionally encountered normally

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THE SYNTHESIS, SOME DERIVATIVES, AND THE METABOLISM OF α,γ -DIKETO-*n*-OCTANOIC ACID

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It is generally believed that in the course of the biological oxidation of fatty acids either mono- or polyketonic acids are formed as intermediates preceding the breakdown to the ketone bodies and other fragments. However, such higher ketonic acids have never been isolated from mammalian tissues, and only a few compounds pertaining to this situation have been synthesized and described. It is apparent that in order to determine the detailed mechanism of fatty acid oxidation to the same degree which has been accomplished for carbohydrate metabolism the chemistry and physiology of such hypothetical intermediate ketonic acids must be approached experimentally.

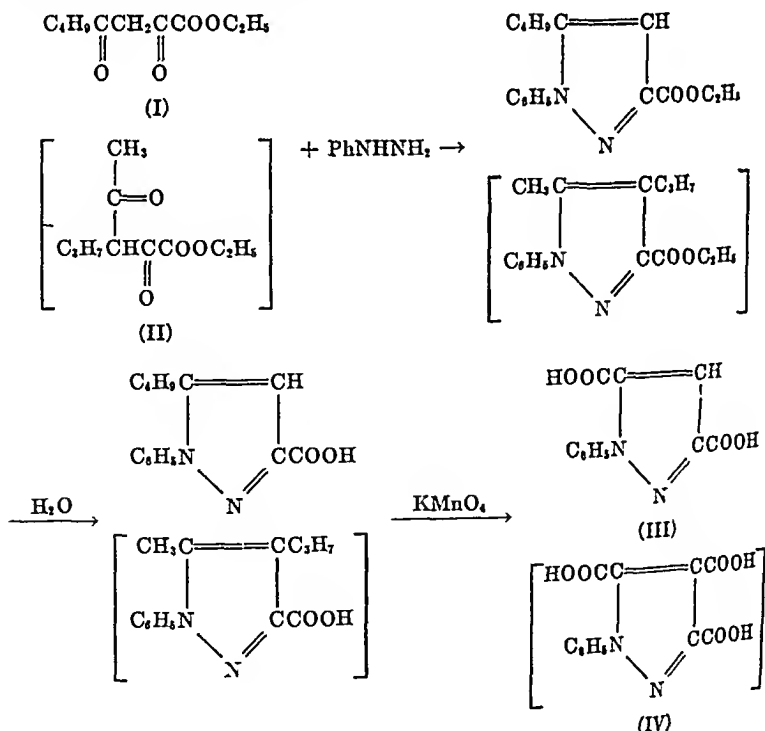
Since an α,γ -diketo acid (acetoacetic acid) has shown marked metabolic activity in the *in vitro* experiments of Krebs and Johnson (1) and in the *in vivo* experiments of the author (2), it was decided to investigate the synthesis and metabolism of an 8-carbon homologue having the α,γ -diketo configuration, i.e., α,γ -diketo-*n*-octanoic acid. Although there is no definite experimental reason at present to suspect the biological occurrence of α,γ oxidation of even chain fatty acids, Jowett and Quastel (3) have pointed out that this mechanism may well obtain in the case of the odd carbon acids, and that the possibility of its occurrence in the case of even carbon acids cannot be dismissed. The choice of the 8-carbon homologue was made, since it invited comparison in biological activity with its parent substance, octanoic acid, which represents the maximum effective chain length for *in vitro* work (due to solubility considerations (3)).

Since this compound has not been previously described, this report will consider first its synthesis, properties, and certain of its derivatives, studies on its intestinal absorption and metabolism will complete the report.

Synthesis, Properties, and Derivatives—Lower homologues have been prepared by the Claisen condensation of ethyl oxalate with the appropriate methyl ketone in the presence of sodium ethoxide, followed by saponification of the ester formed (4-7). Although the velocity of this condensation diminishes considerably with increasing size and complexity of the methyl ketone, it was found possible to effect the condensation of methyl *n*-butyl ketone and ethyl oxalate in 60 per cent yield by running the reaction at

higher temperatures and by recovering the product as the ethyl ester, rather than by the more usual isolation of the sodium derivative, which in this case was found to be quite soluble in the alcoholic medium

That the condensation of methyl η -butyl ketone with ethyl oxalate actually took place with the methyl group, resulting in α,γ -diketooctanoic ester (I), and not with the α -methylene group (which would result in α keto- γ acetylhexanoic ester (II)) was demonstrated by a method successfully employed by Tracy and Elderfield (8) in the case of a lower homologue. The homogeneous ethyl ester formed in the Claisen condensation was allowed to react with phenylhydrazine, forming a substituted pyrazolecarboxylic ester. This was saponified and the alkyl side chain oxidized with alkaline permanganate. If the α,γ -diketo configuration resulted in the original Claisen condensation, then the product of the permanganate oxidation should be 1-phenylpyrazole-3,5-dicarboxylic acid (III) (9), if the branched chain acid resulted, then the product of the oxidation should be 1-phenylpyrazole-3,4,5-tricarboxylic acid (IV) (10). Only the former compound could be isolated, and since the ester formed in the Claisen condensation was homogeneous this is proof of the α,γ -diketooctanoic acid structure (see Formulas I to IV)



Both the ester and the free acid react with hydrazine or substituted hydrazines to form pyrazole derivatives. Tracy and Elderfield (8) have shown that when the ethyl ester of a lower homologue reacts with phenylhydrazine, two isomeric pyrazoles are formed, depending on whether the α - or γ -carbonyl group is initially involved. Only one homogeneous product was grossly apparent with the 8-carbon homologue when semicarbazide or 2,4-dinitrophenylhydrazine were condensed with the free acid. In the case of condensations of the hydrazines with the ethyl ester homogeneous derivatives were isolated, but no special attempt was made to determine the presence of the other isomers nor to determine the configuration of the isolated derivatives.

The free diketo acid was found to be liquid at room temperature and only very slightly soluble in water. It formed readily soluble sodium and potassium salts. The barium, magnesium, calcium, copper, lead, silver, and mercury salts were insoluble as expected. The diketo acid was labile to alkali, decomposing into methyl butyl ketone and oxalic acid in the presence of excess NaOH. At pH 8.0 at 0° a 0.05 M solution showed noticeable decomposition in 2 days, as evidenced by the odor of methyl butyl ketone which developed on standing.

The general method for the synthesis of α, γ -diketo esters was employed in an attempt to synthesize a 20-carbon homologue. A sample of hexadecanone-2 (prepared by the dry distillation (11) of a mixture of the barium salts of acetic and margaric acids, the latter being of questionable purity) was condensed with ethyl oxalate in the presence of sodium ethoxide by using a 24 hour period under a reflux. Elementary analysis of the ester formed agreed with the calculated values within the error of the determinations, but a constant melting point could not be obtained. This was probably due to the contamination of the original margaric acid by lower and higher homologues which would be carried through into the final condensation product as homologous α, γ -diketo esters. The difficulties of detecting and removing such impurities are of course well known. The synthesis of higher homologues is apparently perfectly feasible by this method and rests only on the availability of the appropriate methyl ketones.

Metabolism—The experiments described on the biological utilization of this compound are perhaps not very promising when considered from the point of view of *in vitro* work on surviving tissue slices. The compound (in solution as the sodium salt) was not absorbed readily from the alimentary canal, nor did it affect in any way the basal Q_{O_2} of surviving rat tissue slices. It must be noted that the compound forms almost insoluble salts with calcium and magnesium and for this reason the usual Krebs-Ringer-phosphate buffer was substituted by an isotonic phosphate-saline buffer. That the compound was not freely diffusible into the slices as a probable consequence of this solubility behavior was shown when it was found that broken cell

preparations, such as fine minces and homogenates, could oxidize the compound, albeit slowly, whereas the whole slice showed no extra oxygen uptake in its presence

The compound was found to be decarboxylated by yeast carboxylase at an extremely low rate, and indeed acted as an inhibitor in pyruvate decarboxylation in the same system. Anaerobic experiments with mammalian tissues showed no dismutation effects as described by Krebs and Johnson for pyruvic acid (12)

The failure of the compound to be metabolized to any great extent contrasts sharply with the metabolic activity of acetylpyruvic acid, its lower homologue (1, 2), and may indicate that it is not a normal metabolic. However, caution must be maintained in this regard because the experiments have called attention to perhaps the greatest problem in experimentation *in vitro* on fatty acid oxidation, namely, the technical problem of solubility, diffusion, and physical state of the lipid investigated. Although it is known that liver slices oxidize endogenous 16- and 18-carbon fatty acids (13), those same compounds invariably reduce the basal respiration of slice and cell-free preparations (14) when added to these as their slightly soluble alkali salts. From these considerations, it would perhaps be presumptive to rule out the possibility of α,γ -diketo intermediates on the basis of the exploratory work here reported. It is quite clear, however, that the diketo acid is relatively inert compared to its parent substance, octanoic acid.

EXPERIMENTAL

Synthesis and Derivatives of α,γ -Diketo-octanoic Acid

*Ethyl α,γ -Diketo-*n*-octanoate*—As starting material for the Claisen condensation, *n*-butyl methyl ketone was prepared via the acetoacetic ester synthesis (15), the ethyl ovalate was an Eastman product.

To 2.42 gm of sodium dissolved in 25 ml of absolute ethanol, maintained at boiling temperature under a reflux, a mixture of 10.0 gm of *n*-butyl methyl ketone and 14.6 gm of ethyl ovalate (both dried over Na_2SO_4) was added dropwise with stirring over a period of 30 minutes. After the addition was complete, the turbid mixture was refluxed for 2 hours. The whole reaction mixture was then poured over 100 gm of crushed ice, immediately followed by 10 ml of concentrated H_2SO_4 . The mixture was quickly and thoroughly stirred, the ester collecting as an oily layer. The whole mixture was then extracted with benzene, the extract dried by one shaking with anhydrous Na_2SO_4 , and the benzene removed *in vacuo*. The crude ester was then distilled *in vacuo*, collecting the fraction from 120–145° at 13 mm. The ester after two more distillations was found to boil at 138–139° at 13 mm. The yield of the pure product was 55 to 60 per cent of the theoretical. The

pure ester has a yellow color, resembling the lower homologous ethyl esters in this respect. No other major reaction product could be isolated.

Analysis— $C_{10}H_{18}O_4$. Calculated, C 59.96, H 8.09, found, C 59.78, H 8.29.

Proof of Structure—The ethyl ester was first condensed with phenylhydrazine to yield a pyrazole derivative. A mixture of 2.0 gm. of the ethyl ester formed in the Claisen condensation, 1.3 gm. of phenylhydrazine, and 3 ml. of glacial acetic acid was refluxed for 10 hours, poured on ice, and extracted with ether. The ether extract was dried over Na_2SO_4 and the ether removed *in vacuo*. No attempt was made to separate the possible isomeric pyrazolecarboxylic esters, since their simultaneous presence has no bearing on the final result (8).

The residue was then saponified by refluxing with 1 equivalent of 1 N NaOH for 2 hours. The mixture was filtered hot, acidified, and extracted with ether. The extract was dried and evaporated to yield a yellow oil, which was completely soluble in dilute NaOH and insoluble in dilute HCl.

The crude acid was then subjected to permanganate oxidation. The acid was dissolved in a minimum volume of 1 N NaOH and brought to boiling temperature under a reflux. Through a dropping funnel 4.7 per cent aqueous potassium permanganate was added dropwise, with continuous refluxing, until no more decolorization took place. Approximately 4 to 5 moles of $KMnO_4$ were required. After 2 hours at 100° , the slight excess of $KMnO_4$ was reduced by the addition of H_2O_2 and the hot mixture filtered. The clear, slightly alkaline filtrate was concentrated to a small volume and acidified with concentrated HCl. White needles separated on cooling. The substance was treated with norit and recrystallized to give a pure product having a melting point of 265° ,¹ which was not altered on further recrystallization. The yield was 1.3 gm., or 57 per cent calculated from the original ethyl ester.

The two possible pyrazole derivatives which might result from this series of reactions (see Formulas I to IV) are 1-phenylpyrazole-3,5-dicarboxylic acid, m.p. 266° (9), and 1-phenylpyrazole-3,4,5-tricarboxylic acid, m.p. 138° (10). The obvious identity of the product obtained with the first possibility was proved by synthesis of the former from ethyl acetopyruvate (9). The melting point of the authentic sample was 266° , the mixed melting point with the product obtained was 265° . Since the ester formed in the Claisen condensation was homogeneous, only one product, α,γ -diketo-*n*-octanoic ester, was formed.

*α,γ -Diketo-*n*-octanoic Acid*—The free acid was obtained by saponification of the ester. To 50 ml. of 1.0 N NaOH were added 5 gm. of the ethyl ester and the mixture shaken vigorously for 3 minutes, forming a slightly turbid

¹ All melting points have been corrected for stem exposure.

yellow solution. It was immediately acidified with 10 N H_2SO_4 , yielding a turbid white emulsion which on standing and cooling in ice water separated into two layers. The lower layer is the crude free acid, which has a melting point just below room temperature. The lower layer was separated and extracted several times with small portions of warm water to remove traces of oxalic acid and the ketone. The free acid was then carefully brought into solution as the sodium salt by suspending it in a small quantity of water with high speed stirring and adding dropwise 4 N NaOH (carbonate-free) until solution was effected. The pH should not rise above 8 in order to avoid hydrolysis to oxalic acid and butyl methyl ketone. The solution was then shaken with a small amount of norit and filtered. An excess of saturated barium acetate solution was then added. The barium salt separated as slightly yellow needles from dilute solutions or in an amorphous form from strong solutions. After standing overnight at 0° it was filtered off, washed with a small volume of ice water, and allowed to dry at room temperature. It was then washed with a very small amount of cold ether and dried over P_2O_5 *in vacuo*. The yields averaged 40 to 50 per cent and could be improved by reworking the mother liquor of the barium salt and the ether washings.

Analysis— $\text{C}_{16}\text{H}_{32}\text{O}_6\text{Ba}$. Calculated, Ba 28.6, found, Ba 28.4

The free acid could not be readily purified by vacuum distillation, since extensive decomposition usually resulted. Likewise, recrystallization at low temperatures was found inconvenient although possible. The barium salt was soluble in ether and absolute alcohol.

To obtain the free acid in solution as the neutral sodium salt (as for the biological experiments described later) a weighed quantity of the barium salt was suspended in water and the equivalent amount of sodium sulfate solution was added. The mixture was then homogenized either in a Waring blender or the device of Potter and Elvehjem (16) at intervals over the course of a day. The barium sulfate was centrifuged off and the supernatant shaken with acid-washed norit, filtered, neutralized, and made up to volume, yielding a clear yellow solution.

Alkaline Degradation of Free Acid—The free acid was found to undergo alkaline hydrolysis to *n*-butyl methyl ketone and oxalic acid. The free acid was refluxed with 3 equivalents of 2 N NaOH for 2 hours. Treatment of a portion of the neutralized mixture with a 0.1 per cent solution of 2,4-dinitrophenylhydrazine in 2 N HCl yielded the 2,4-dinitrophenylhydrazone of *n*-butyl methyl ketone, m.p. 106° , authentic sample, m.p. 106° , mixed m.p. 106° . The oxalic acid formed was identified as its calcium salt and subsequent permanganate titration. At pH 7.4 the sodium salt is stable in solution at 0° for several days. The hydrolysis can easily be detected by the odor of the methyl butyl ketone formed.

*Copper Ethyl α, γ -Diketo-*n*-octanoate*—2 gm of the purified ester were dissolved in 20 ml of ethanol. To this solution was added 1 equivalent of cupric acetate dissolved in the minimum required volume of boiling water. A dark green precipitate formed which was filtered off and dried in air. The copper derivative was found to be soluble in organic solvents and was quite volatile, both indications of a chelate ring structure. It was recrystallized from an ethanol-water mixture, yielding dark green needles. The compound melted at 135–137° after some discoloration. Yield, 80 per cent.

Analysis— $C_{18}H_{28}O_4Cu$. Calculated, Cu 13.77, found, Cu 14.00.

1-(2,4-Dinitrophenyl)-5(3)-butylpyrazole-3(5)-carboxylic Ethyl Ester—1 gm of ethyl α, γ -diketooctanoate in alcoholic solution was treated with 1 gm of 2,4-dinitrophenylhydrazine in 30 ml of ethanol plus 5 ml of concentrated HCl. A yellow precipitate formed immediately. After the mixture was heated for 30 minutes and cooled, the precipitate was filtered off and washed with ethanol and then water. It was recrystallized from ethanol, to give yellow needles melting at 186–187°. Yield, 74 per cent.

Analysis— $C_{18}H_{19}O_7N_4$. Calculated, C 53.05, H 5.01, found, C 52.98, H 5.22.

1-(2,4-Dinitrophenyl)-5(3)-butylpyrazole-3(5)-carboxylic Acid—The sodium salt of the keto acid in aqueous solution was treated with 0.1 per cent 2,4-dinitrophenylhydrazine in 2 N HCl, yielding a flocculent precipitate composed of pale yellow needles. The compound was recrystallized from toluene. It began to decompose at 185° with volatilization and formed a clear melt at 204°. Yield, 89 per cent.

Analysis— $C_{18}H_{17}O_7N_4$. Calculated, C 50.28, H 4.22, found, C 50.22, H 4.12.

1-Carboxamide-5(3)-butylpyrazole-3(5)-carboxylic Acid—The sodium salt of the keto acid in aqueous solution formed a voluminous white precipitate in almost quantitative yield when treated with aqueous semicarbazide hydrochloride. This precipitate formed almost immediately in the cold or on slight warming, and was so insoluble it could be used as a roughly accurate gravimetric estimate of the concentration of the acid. The compound was filtered off and dried *in vacuo* over H_2SO_4 . It was found to be labile to heat and water, as was the lower homologue previously described (17). The compound was obtained analytically pure without further treatment. Decomposition began at 80–82° and a clear melt was formed at 160–165°.

Analysis— $C_7H_{13}O_3N_3$. Calculated, C 51.18, H 6.20, found, C 51.10, H 6.33.

5-Butylpyrazole-3-carboxylic Acid—This was formed by hydrolysis of 1-carboxamide-5-butylpyrazole-3-carboxylic acid by a method previously reported (17). The compound was recrystallized from water to yield

colorless prisms, which melted at 166–167° after drying over H_2SO_4 *in vacuo*

Analysis— $\text{C}_8\text{H}_{12}\text{O}_2\text{N}$ Calculated, C 57.12, H 7.19, found, C 57.01, H 7.28

The compound was also obtained from α, γ -diketooctanoic acid by reaction, in aqueous medium, with hydrazine sulfate. Its identity was confirmed by elementary analysis and mixed melting point tests.

Metabolism

In these experiments the sodium α, γ -diketooctanoate employed was that obtained from a triply precipitated barium salt and in most cases some made from the singly precipitated barium salt was used also for comparison. There was no discernible difference in the behavior of these preparations in the biological experiments, minimizing the possibility of interference by trace impurities.

Absorption from Intestinal Tract—Four rats, weighing 150 to 200 gm, were fasted for 24 hours and each then given 5.0 ml of 0.05 M sodium diketooctanoate by stomach tube. The urine and feces were collected separately for the next 12 hours and the animals were then sacrificed. The entire alimentary canal of each rat was then removed, emptied, and washed out with a stream of warm water. The feces, intestinal contents, and washings from all four rats were then combined, homogenized in a Waring blender, acidified, and extracted with ether. A similar group of four rats was treated in identical manner, 0.05 M sodium bicarbonate being substituted for the keto acid solution, and the feces and intestinal contents worked up in the same way.

The ether extracts were evaporated to dryness *in vacuo* and taken up in a few ml of warm ethanol. Saturated sodium bicarbonate solution was added with stirring until the solution was neutral. It was then filtered and treated with an excess of solid semicarbazide hydrochloride and sodium acetate.

The characteristic insoluble derivative of α, γ -diketooctanoic acid appeared in the extract from the animals fed the compound. After standing at 0° for several hours it was filtered off, washed with water, and dried *in vacuo*. Its melting point and neutralization equivalent (in alcohol) showed it to be quite pure. Its weight corresponded to a recovery of 67 per cent of the keto acid administered.

It was converted to 5-butylpyrazole-3-carboxylic acid by boiling with acid (17) and recrystallized. Mixed melting point tests proved the identity of the compound.

The control extract yielded 3 to 4 mg of amorphous material after treatment with semicarbazide. It was soluble in excess water and was not iden-

tical with the derivative of α, γ -diketooctanoic acid or that of butyl methyl ketone, as proved by mixed melting point tests

Treatment of the pooled urines of the two groups with semicarbazide yielded no insoluble material

The only toxic manifestation noted after administration of the diketo acid was a slight diarrhea

Reaction with Yeast Carboxylase—The method of Krebs and Johnson (1) was used in a determination of the degree of decarboxylation of α, γ -diketooctanoic acid, pyruvic acid, and an equimolecular mixture of the two by a Lebedev extract of brewers' yeast. The compounds were tested in a final concentration of 0.01 M. In 4 hours only 3 per cent of the theoretical yield of CO_2 could be obtained from α, γ -diketooctanoic acid. Pyruvic acid was completely decarboxylated in 30 minutes by the same preparation. When both compounds were present together, the decarboxylation of pyruvic acid was inhibited 98 per cent.

TABLE I

Oxidation of α, γ -Diketooctanoate by Minced and Homogenized Liver

Temperature 38°, gas phase, air, 0.2 ml. of 0.05 M substrate or saline tipped into 1.0 ml. of 10 per cent suspensions of minced or homogenized liver. The center well was furnished with KOH. Time, 1 hour.

Preparation	Substrate	Oxygen uptake
		<i>c mm</i>
Mince	—	88
	+	100
Homogenate	—	70
	+	84

Oxidation by Tissue Slices—In these experiments rat tissue slices, prepared in the usual manner (8 to 20 mg. of dry weight) were equilibrated with 2.0 ml. of phosphate-saline buffer of pH 7.4 at 38° in Warburg vessels. The contents of the side bulb (either saline or 0.05 M sodium diketooctanoate) were then tipped in and measurements of oxygen uptake taken over a 2 hour period. The results, expressed as Q_{O_2} values (c mm. of O_2 taken up per mg. of dry tissue per hour), showed no significant difference between control tissues and those in the presence of the diketo acid. Either the acid is not oxidized by the tissues or it is unable to diffuse into the cells. The tissues examined were rat brain, liver, heart, kidney, diaphragm, and testis. Buffers containing Mg^{++} or Ca^{++} precipitate the diketo acid readily, even from very dilute solution.

Anaerobic Experiments with Rat Liver Slices—The anaerobic dismutation of α -keto acids, especially pyruvic acid, to yield CO_2 has been studied

extensively by Krebs and Johnson (12). The participation of α,γ diketooctanoic acid in such reactions was investigated with the methods developed by the above authors. No extra CO_2 was produced anaerobically when the diketo acid was present alone and the yield of CO_2 from pyruvate and oxalacetate was sometimes slightly depressed by the presence of the diketo acid, although the magnitude of such inhibition was near the inherently large experimental error.

Oxidation by Broken Cell Preparations—When the diketo acid was incubated at pH 7.4 with 10 per cent suspensions of minced or homogenized rat liver in phosphate buffer, invariably a slight increase in oxygen uptake over the controls was observed. The increase was not large but was definitely evidence of some oxidative activity. A typical experiment is given in Table I.

SUMMARY

1 The synthesis of α,γ -diketooctanoic acid was achieved by using an extension of available methods for lower homologues. The structure of the product was demonstrated by appropriate experiments.

2 Several derivatives of pyrazole nature were obtained from the free acid and ethyl ester by reaction with substituted hydrazines.

3 The degree of intestinal absorption of sodium α,γ -diketooctanoate was found to be quite small.

4 Experiments with surviving rat tissue slices showed no evident biological utilization of the compound, possibly due to its low diffusibility into the slices. In support of this interpretation, it was found that broken cell preparations of rat liver were able to oxidize the compound at a low rate.

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ACETYL PHOSPHATE CHEMISTRY, DETERMINATION, AND SYNTHESIS*

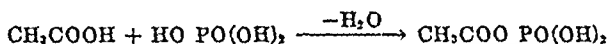
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During experiments with bacterial enzyme preparations, it was observed that the oxidation of pyruvate depended on the presence of phosphate (1). Therefore it was to be expected that intermediately a phosphorylated compound was formed. For a long time, however, we were not able to detect a fixation of phosphate accompanying this reaction. Eventually the possibility was realized that an unusually labile compound might be formed. Recent observation on the formation of an acyl phosphate in the course of fermentation (2) pointed to acetyl phosphate as a possible precursor of acetic acid. The further study of the problem led to a demonstration, first, of the formation of a very labile organophosphate (3, 4) and, lately, to its identification as monoacetyl phosphate (5).

The progress of this work was greatly facilitated when we had acquired a more intimate knowledge of this peculiar substance, acetylphosphoric acid, the anhydride of acetic acid and one of the hydroxyls of phosphoric acid.



Our studies on the chemistry and determination of acetyl phosphate will be described in detail in this paper. Subsequent communications will deal with the biochemistry of acetyl phosphate.

Survey of Properties of Acetyl Phosphate

It will facilitate the understanding of the precautions necessary for isolation, synthesis, and determination of the compound if, first, its properties are surveyed in a general manner. Considering the fact that the configuration of acetyl phosphate is analogous to acetyl chloride or acetic anhydride, the acetyl phosphate appears remarkably stable. At room temperature and in neutral solution its half life time is several hours. In the frozen state, at -35° , solutions may be kept for weeks without appreciable deterioration. On either side of the neutral region the stability diminishes, first slowly, then rapidly. Fig. 1 shows in detail the dependence of stability on pH, between 4 and 10. Acetate, veronal, and ammonia buffers were used.

* This work was supported by a grant from the Commonwealth Fund.

The stability maximum lies distinctly on the acid side, between pH 5 and 6. Accordingly, it has been our experience on handling the compound that acid reaction is far better tolerated than alkaline. This is especially true for impure preparations when often an uncontrollable labilization occurred through contaminants. The relatively great stability at acid reaction makes it possible to use, with certain precautions, trichloroacetic acid for deproteinization. It may be mentioned here that if hydrogen

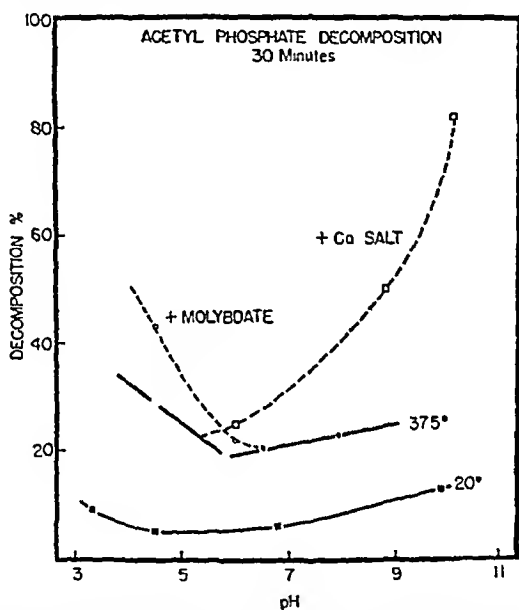


FIG 1 Dependence of decomposition of acetyl phosphate on pH. In the experiments reproduced, in the uppermost right curve 0.5 per cent calcium chloride was present, in those of the uppermost left, 0.1 per cent ammonium molybdate had been added.

sulfide is used to remove lead or silver ions it invariably results in almost complete loss of acetyl phosphate.

Of practical and theoretical interest is the enormous acceleration of acetyl phosphate breakdown in acid solution with molybdate. In Fig 2 the rates of hydrolysis with 0.5 N mineral acid are compared with and without molybdate. In plain acid the hydrolysis was followed by the precipitation method, while the curves for molybdate in 0.5 N acid (Fiske Subbarow reagent (6)) were obtained by direct colorimetric observation of the breakdown. Both procedures will be described in detail later in this

paper From the data represented in the curves the hydrolysis constants (time in minutes) were calculated to be 2.78×10^{-2} in plain acid at 40° , and to be 3.5×10^{-1} in the Fiske-Subbarow reagent at 23° The almost explosive breakdown of acetyl phosphate with the Fiske-Subbarow reagent is due, therefore, to the presence of molybdic acid rather than to mere acidity

This acceleration by molybdic acid is comparable to the earlier observed effects on creatine phosphate (7) and phosphoglyceryl phosphate (2) breakdown The very general molybdate catalysis of the breakdown of organo-

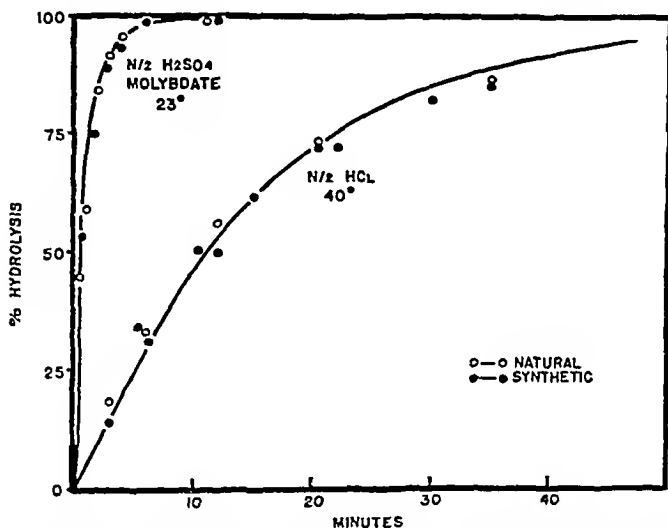


Fig 2 Hydrolysis of natural and synthetic acetyl phosphate in 0.5 N mineral acid with and without molybdate

phosphates may tentatively be attributed to the removal of inorganic phosphate by the formation of a complex ion This would be analogous to the acceleration, shown in Fig 2, of alkaline acyl phosphate decomposition through calcium ions, due obviously to the removal of inorganic phosphate by precipitation

The composite hydrolysis curves of Fig 2 represent data of both natural and synthetic compounds The points lie reasonably close to the drawn average curve This practical identity of the hydrolysis function provided at an early stage welcome corroborative evidence for the identity of the naturally formed compound with acetyl phosphate With reference to the natural product, the molybdate curve was derived from phosphate deter-

minations in trichloroacetic acid extracts of *Bacterium delbrückii* preparations which had been incubated with pyruvate (4), the acid decomposition curve refers to crude silver fractions of the primary oxidation product of pyruvic acid

Determination of Acetyl Phosphate

Principle of Method—With the molybdate reagent of Fiske and Subbarow complete decomposition of acetyl phosphate occurs at room temperature in less than 10 minutes (*cf* Fig 2). Therefore, direct colorimetry yields an *apparent* phosphate value which represents the sum of acetyl and inorganic phosphate and includes also creatine phosphate if present (*cf* below). To separate the labile organic and the inorganic phosphate the differential solubility of the calcium salts is used. *True* inorganic phosphate is precipitated with alcoholic calcium chloride solution, leaving the soluble calcium acetyl phosphate in the supernatant. Addition of alcohol is needed to precipitate quantitatively calcium phosphate at as low a pH as 8. Work at a low pH is necessary because, with a stronger alkaline reaction, acetyl phosphate is unstable especially in the presence of calcium ion (*cf* Fig 1). The difference between apparent and true inorganic phosphate is regarded as acetyl phosphate. If necessary, acyl phosphates may be differentiated from creatine phosphate through their lability to alkali

Procedure

Reagents—

For Deproteinization—3 to 5 per cent trichloroacetic acid

For Neutralization—A mixture of 100 ml of concentrated ammonia and 40 ml of glacial acetic acid is made up to 1 liter, and to that are added 100 ml of 0.4 M bicarbonate solution. Mallinckrodt's ammonium "carbonate," a mixture of ammonium carbamate and bicarbonate, according to the label, was used preferentially, but any bicarbonate will do.

For Phosphate Precipitation—A 3.3 per cent solution of anhydrous calcium chloride in 33 per cent ethanol

Deproteinization—The sum of inorganic and acetyl phosphorus (direct colorimetry = total P) per sample was usually 0.5 to 1.5 mg with 10 to 100 per cent acetyl P. One-twelfth of the total, or 40 to 120 γ , was used generally for individual P determinations. Three identically marked 15 ml test-tubes are prepared and kept thoroughly chilled. The third tube contains a drop of thymol blue. The experimental samples, generally 1.5 to 2 ml, are cooled by shaking the vessel in ice water for 1 to 2 minutes, and 5 ml of ice-cold trichloroacetic acid are added. The mixture is shaken vigorously and at once is poured over into the first tube. The protein precipitate is centrifuged in chilled centrifuge cups. The use of a cooled

centrifuge was found unnecessary 2 to 3 minutes centrifugation was found sufficient The supernatant is poured into the second tube

Neutralization—Exactly 0.5 ml of trichloroacetic acid extract is transferred (a) to the third tube for precipitation of true inorganic phosphate, and (b) to a volumetric flask for direct colorimetric determination To sample (a) the reagent for neutralization is added quickly from a burette Local alkalization should be carefully avoided After the indicator has changed from red to yellow, the less dangerous pH zone above 4 is reached The final adjustment to a grayish blue color, corresponding to pH 8, may then be carried out more leisurely Usually about 0.3 ml of reagent is needed The exact amount may be conveniently determined beforehand in a blank

Precipitation of Calcium Phosphate—To the neutralized sample, now about 0.8 to 1 ml, 2.5 ml of alcoholic calcium chloride solution are added When the material is mixed, the color of the indicator usually turns more yellowish but should retain a bluish tinge To make the precipitate bulkier, especially needed if only small amounts of P are present, 0.15 ml of 0.04 M bicarbonate solution is added dropwise The calcium precipitate, phosphate plus carbonate, is quickly centrifuged off 1 to 2 minutes is sufficient The supernatant is decanted carefully, adhering fluid washed off with 2.0 ml of the alcoholic calcium chloride solution, and the precipitate is recentrifuged without stirring

Here the procedure may be interrupted for any length of time Up to this point, however, not more than 15 to 20 minutes should be taken from the addition of trichloroacetic acid

Colorimetry—The calcium precipitate is dissolved in 0.5 ml of 0.5 N hydrochloric acid and quantitatively brought into a volumetric flask of convenient size Phosphorus is determined colorimetrically by the procedure of Fiske and Subbarow (6), with the only modification (*cf* (8)) being that after addition of the reagents and filling up to volume all flasks were submersed for 7 minutes in a bath of 37° A parallel, direct phosphate determination is carried out on the second 0.5 ml, sample (b) The difference between directly determined and precipitated phosphate, the labile phosphate, represents acetyl phosphate

Supplementary Remarks

Deproteinization—It was found in preliminary experiments that an acidity of below pH 1.8, just acid to cresol red, was necessary to bring about a complete precipitation of protein with trichloroacetic acid A 3 per cent trichloroacetic acid solution in the proportion described is just sufficient to give this acidity, if the buffering capacity of the sample is not extraordinarily high Higher concentrations of trichloroacetic acid have been used, up to 5 per cent, without causing serious decomposition

Neutralization—Thymol blue is recommended as the indicator, because its two color changes, at pH 3 from red to yellow and at pH 8 from yellow to blue-purple, just enclose the zone of greatest stability of acetyl phosphate. Red and purplish blue, therefore, both indicate danger of decomposition, while yellow indicates comparative safety. As it is desirable to shorten as much as possible the exposure to the initial low pH of about 1.5, for a larger series of parallel determinations a fractionated type of neutralization was adopted. Two-thirds of the total amount of neutralization reagent needed, or about 0.2 ml, is measured into the tubes which shall receive the 0.5 ml of trichloroacetic acid filtrate. This brings the added fluid instantaneously to a pH of around 5, with the indicator a bright yellow. At this pH a short delay is not harmful, final neutralization to the more dangerous pH of 8 can be conveniently timed.

Calcium Phosphate Precipitation—Although plain calcium acetyl phosphate is still soluble in alcohol up to 80 per cent, a coprecipitation frequently occurs with higher alcohol concentrations when inorganic phosphate is present in larger amounts. For this reason it is advisable to keep the alcohol concentration low. This furthermore avoids a contamination of this fraction with calcium salts of creatine phosphate or other organic phosphates which are insoluble at higher alcohol concentrations.

The accuracy of the method obviously depends on the completeness of precipitation of calcium phosphate within the limiting conditions imposed by the instability of the organic phosphate. With plain phosphate solutions, in the range of 10-7 per sample and upwards, we get consistently complete recovery. For work at lower levels it is advisable to decrease the volume of the reagents rather than the concentration of phosphate. With trichloroacetic acid filtrates occasionally differences were found between apparent and true phosphate of 2 to 3 per cent, the reliability of which appeared doubtful. A difference of below 5 per cent, although it may be of significance, should be considered with due caution.

Simultaneous Determination of Acetyl and Creatine Phosphate

Creatine phosphate is thoroughly stable in alkaline solution. In all current methods for its determination, therefore, strongly alkaline reagents may be and are used for the separation of inorganic phosphate. Since the strong alkali completely mineralizes acetyl-bound phosphorus, acetyl phosphate is carried into the inorganic fraction (cf (4)). Therefore, with both acetyl and creatine phosphates present, the addition of a creatine phosphate determination, yielding the sum of acetyl and inorganic phosphate, will furnish the third value needed to calculate the three phosphate fractions, acetyl, creatine, and inorganic phosphate.

Direct Determination of Acetyl-Bound Phosphate

In exchange experiments with radioactive phosphate, to be described in a subsequent communication, the determination of acetyl-bound phosphate directly rather than by difference appeared desirable. For this purpose the acetyl phosphate, which stays in the supernatant of the calcium phosphate precipitate, is decomposed with strong alkali and determined as calcium phosphate. The procedure is carried through as described. The supernatant of the calcium precipitate, however, instead of being discarded, is collected quantitatively. Then, normal sodium hydroxide is added, a volume one-tenth that of the supernatant being used, and the alkaline fluid is kept for 15 minutes at 37°. The precipitate is centrifuged off, dissolved in acid, and phosphate is determined as usual.

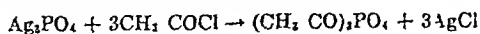
Since creatine phosphate is stable in alkali, this method may also be used to distinguish between creatine and acetyl phosphate.

Direct Colorimetric Determination

Although the decomposition of acetyl phosphate is rather rapid in acid molybdate, the mineralization of acetyl-bound phosphate may be followed colorimetrically. The procedure is analogous to that used by Eggleton and Eggleton (9) and Fiske and Subbarow (7) in their early work on creatine phosphate. Two volumetric flasks are prepared in parallel, with standard and experimental phosphate respectively as well as aminonaphtholsulfonic acid-sulfite reagent (6), and filled up to a volume just allowing space for subsequent addition of molybdate reagent. Molybdate finally is added to both flasks simultaneously and after inverting the color reading is started as soon as possible. Owing to progressive liberation of acetyl-bound phosphate, there is a color increase during the first few minutes. In this manner the decomposition with molybdate-acid mixture was measured as shown in the upper curve of Fig. 2. The method allows a check, by direct observation, upon the presence of labile organic phosphates.

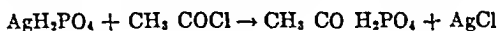
Synthesis of Acetyl Phosphate

Polyacetyl phosphates may be obtained easily by the reaction between yellow trisilver phosphate and acetyl chloride



From polyacetyl phosphate, however, it was not possible to derive a homogeneous preparation of the biologically interesting mono compound. A relatively laborious synthesis of monoacetyl phosphate was described by Lynen (10), using the detour over monosilver dibenzyl phosphate with subsequent elimination of the benzyl groups by catalytic hydrogenation. This

time-consuming detour has now been made unnecessary by using, instead of monosilver dibenzyl phosphate, monosilver dihydrogen phosphate



Although a silver phosphate of the above composition is not known as a well defined compound, a mixture of 1 mole of trisilver phosphate and 2 moles of phosphoric acid yields with acetyl chloride under our conditions up to 70 per cent of the theoretically possible amount of monoacetyl phosphate. Such a direct condensation may be of general use in procedures of an analogous character, as, for example, Cori's chemical synthesis of glucose-1-phosphate (11)

The success of this procedure depends largely on thorough cooling and quick manipulation. A cold room is not needed, but all the vessels, funnels, and fluids used during the following operations have to be refrigerator-cold, and the receiving vessels have to be kept in an ice bath.

Preparation of Monosilver Phosphate—In a 250 ml round bottom, dry centrifuge bottle (Pyrex, catalogue No 1260), 32 gm of thoroughly dried silver phosphate¹ (76 mm of salt with 230 milliequivalents of silver) and 11 ml of 90 per cent phosphoric acid (Mallinckrodt) (176 mm) are well mixed. Some heat is evolved, and the bright yellow color of the trisilver phosphate changes to a gray. The sirupy mixture largely solidifies on cooling. The mass is finely distributed in 20 to 25 ml of ether until a homogeneous light gray suspension is obtained.

Acetylation—17 ml (240 mm) of acetyl chloride mixed with an equal volume of ether are dropped from a separatory funnel into the freshly prepared suspension. The bottle containing the silver phosphate is kept in ice water and is agitated continuously. From time to time the addition of acetyl chloride is interrupted. The bottle is removed from the ice and shaken more vigorously to break up clumps of silver chloride. About 10 minutes should be allowed for the addition of acetyl chloride in order to avoid excessive rise in temperature. After the total amount has been added, the bottle is stoppered and is shaken for 10 more minutes at room temperature.

Extraction and Partial Neutralization—The ether mixture contains now acetylphosphoric acid, varying amounts of free acetic and phosphoric acids, and some hydrochloric acid. The suspension is brought over into a liter Erlenmeyer flask, some ether being used for washing. The flask is

¹ To prepare silver phosphate dissolve 100 gm of silver nitrate in 500 ml of water and add gradually to 1200 ml of a hot solution of 520 gm of dibasic sodium phosphate dodecahydrate. Boil, filter, and dry in an oven overnight.

cooled carefully, and a chilled solution of molar sodium carbonate* is added in portions, whereby the ether layer conveniently prevents excessive foaming. Neutralization is interrupted when, after the addition of 120 to 140 ml of carbonate solution, a pH of 3 to 3.5 is reached. The color change of universal indicator paper from bright red to red-orange was used, or the change of Congo paper from blue to purplish. Now, to remove silver chloride both the ether and aqueous solution are sucked through a Buchner funnel. (A little Celite Analytical Filter-Aid, Johns-Manville, is used to tighten the filter paper.)

Extraction of Acetic Acid and Partial Removal of Inorganic Phosphate—To avoid a subsequent disagreeable contamination with silver acetate, the free organic acid is removed at this stage by ether extraction. At pH 3 to 3.5 the acetic acid is mainly un-ionized and easily extractable. Acetylphosphoric acid, however, is present as an acid salt and does not leave the water layer. Its decomposition-pH curve, shown in Fig. 1, indicates that at this range only a negligible decomposition will occur in a cold solution. To extract the acetic acid it is sufficient to shake twice for 2 minutes with 3 times the volume of ether.

At this stage we find between two-thirds and one-half of the total phosphorus acetyl-bound. To complete the neutralization, a few drops of phenol red are added, and under constant shaking 33 per cent sodium hydroxide is run in slowly until the color turns reddish (20 to 22 ml). Care should be taken to avoid local overalkalinization which easily may cause appreciable decomposition. Just after a pH of 7 is reached, crystals of sodium phosphate generally start to separate from the chilled solution, and, on shaking, the flask may suddenly be filled with a mass of crystals. The crystallization of sodium phosphate from this concentrated solution offers a convenient method of removing phosphate. To drive crystallization as far as possible the solution was frozen and kept so for at least an hour at -35° . Here the preparation may be interrupted conveniently. In frozen neutral solution we have kept acetyl phosphate for days without detectable loss. The freezing is a convenient but not an essential operation. Without freezing at this stage the removal of inorganic phosphate is less efficient, and the preparation has to be continued immediately.

To separate the sodium phosphate crystals from the acetyl phosphate solution the frozen solution is thawed slowly, broken up as soon as possible, and transferred still partly frozen to a Buchner funnel. The crystal mass is pressed together on the funnel and sucked dry. The temperature in the

* A sodium carbonate solution of this concentration is oversaturated at refrigerator temperature, and to avoid a precipitation of crystals it should be kept in the refrigerator only 1 to 2 hours prior to use.

crystal mass should not rise above -5° , at which temperature the crystals start to melt. Analysis has shown that practically no acetyl phosphate remains on the filter. An aliquot is taken now from the filtrate, and chloride and inorganic phosphate are determined. While these analyses are carried out, the solution is again frozen. Chloride was determined by thiocyanide titration.

Precipitation and Isolation of Silver Salt—The procedure which follows is similar to that of Lynen (10) who first showed that silver acetyl phosphate crystallizes easily from a solution containing excess silver ions. First the remaining inorganic phosphate and the chloride, the silver salts of which are much less soluble than silver acetyl phosphate, are removed with a slight excess of silver nitrate. The solution is adjusted to pH 7 to 8, and 25 per cent silver nitrate solution is added in an amount exceeding by 10 per cent the silver equivalent calculated from phosphate and chloride determinations (about 10 to 25 ml are needed). Depending on the amount of inorganic phosphate present, a more or less pronounced shift to the acid side occurs, and the pH has to be readjusted. The yellowish white precipitate of mixed silver phosphate and chloride is filtered off by suction, the filter paper having been tightened with Celite. If all the phosphate and chloride has been removed, the addition of a few drops of 25 per cent silver nitrate produces a precipitate which dissolves readily on shaking to a bluish white cloud. The precipitation is continued cautiously with 25 per cent silver nitrate solution. The total amount of silver salt added should be twice that necessary to form disilver acetyl phosphate, usually amounting to around 120 ml of silver salt solution. The copious precipitate is separated from the mother liquid by filtration through a Buchner funnel; washed on the funnel once with a little ice-cold water and twice thoroughly with small amounts of 33 per cent alcohol. All possible adherent water is removed on the funnel by washing twice with alcohol and twice with ether. The product is dried finally in a vacuum over phosphorus pentoxide. Protected from water and from light, silver acetyl phosphate has been kept for years without decomposition.

The yield is 13 to 16 gm and already the analysis of this product conforms almost exactly with the values calculated for disilver monoacetyl phosphate

$C_2H_3O_2PAg$	Calculated	Acetic acid 16.9, P 8.8, Ag 61.0
353.8	Found	" " 17.4, " 8.7, " 61.1
		(No inorganic P)

The acetic acid residue was determined by titration with sodium hydroxide of the steam distillate from an acidified solution of the compound.

If this is not a thoroughly crystalline compound, the crude salt is dis-

solved with slightly less than its equivalent of 0.25 *N* sodium chloride solution. By dropwise addition of silver nitrate the solution is tested for the presence of inorganic phosphate, a little of which is usually formed during the previous manipulations. As long as the precipitate is yellowish, it is discarded. The subsequent isolation is carried out as described.

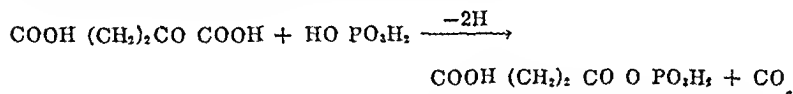
Synthesis of Other Acyl Phosphates

Higher Fatty Acids—When the chlorides of propionic and butyric acids are used instead of acetyl chloride, the corresponding acyl phosphates were prepared in the manner described.

$C_3H_5O_2PAg$	Calculated	Propionic acid	20.1, P 8.4, Ag 58.7
367.8	Found	" "	19.7, " 8.7, " 59.0
$C_4H_7O_2PAg$	Calculated	Butyric acid	23.1, P 8.1, Ag 56.5
381.8	Found	" "	23.4, " 7.9, " 56.4

This series could be easily extended. Because the silver salts or the free acids become more insoluble with increasing chain length, special care has to be taken to extract the free fatty acids as far as possible before precipitation of silver acyl phosphate. Propionyl and butyryl silver phosphates crystallized rather easily, even without reprecipitation. No striking change in the character of the acyl phosphate bond was observed with the lengthening of the carbon chain.

Succinyl Phosphate—The coupling of ketoglutarate oxidation with phosphorylation (12, 13) makes it likely that a fixation of phosphate, yielding succinyl monophosphate, may occur in the following manner:



Therefore, a synthesis of succinyl phosphate was desirable. It was anticipated that the presumably physiological monophosphate could hardly be obtained from succinyl dichloride, the only known chloride of succinic acid, in a direct reaction. It proved, however, possible to obtain by the action of an excess of succinyl dichloride on silver phosphate a mixture of mono and diphosphoryl succinates utilisable for preliminary experiments.

The only major change in the procedure is the use of ethyl acetate rather than of ether for the extraction of free succinic acid. So far the best results were obtained with the following proportions: 20 gm of silver phosphate (50 *mM*) were mixed with 3.3 ml of 90 per cent phosphoric acid (50 *mM*) and 12 ml of ether and the mixture then was treated with 8.3 ml (75 *mM*) of succinyl chloride in 15 ml of ether. The cooling has to be efficient, particularly on addition of the first portions of succinyl chloride. The

total amount was added in about 3 minutes. Thereafter the vessel was removed from the ice bath and shaken at room temperature until heat production ceased. The subsequent treatment was the same as described for acetyl phosphate. 5.5 gm were obtained of a compound which analyzed to 6.6 per cent labile organic and 1 per cent inorganic P, 58.2 per cent silver, and 16 per cent succinic acid.

Succinic acid was determined manometrically with muscle enzyme (14). The ratio 1.57 of organic phosphorus to succinic acid indicates that about 40 per cent of the succinic acid is present as mono- and 60 per cent as diphosphate.

Stability and behavior of this compound are very similar to those observed with other acyl phosphates. Part of the preparation has been turned over to Dr S. Ochoa who is engaged in the study of enzymatic ketoglutarate oxidation (13). In our own experience, all labile phosphate was mineralized rapidly with tissue extracts. The rate of decomposition here was only slightly inferior to that found with acetyl phosphate. In general, we find that acyl phosphates are rapidly decomposed by tissue extracts, a reaction which is inhibited by fluoride and in some instances is sensitive to hydroxylamine.

SUMMARY

1. The properties of acetyl phosphate are studied, especially its stability under various conditions.

2. A method for the determination of acetyl phosphate is worked out.

3. In a relatively simple manner, monoacetyl phosphate was synthesized by interaction of monosilver dihydrogen phosphate and acetyl chloride.

The preparation is reported of propionyl, butyryl, and succinyl phosphates.

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DETERMINATION OF URINARY AMINO NITROGEN BY THE COPPER METHOD*

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The problem of having to perform numerous urinary amino N determinations under limited facilities led us to attempt the adaptation of the copper method of Pope and Stevens (1) for the estimation of the free amino acids to the urine, since it appeared to be simpler and less time-consuming than the gasometric (2), colorimetric (3), or formol titration (4) techniques. These authors have shown that their method compares favorably with the Van Slyke procedure (5) for protein hydrolysates and amino acids. Most encouraging to our purpose is their statement that ammonia, which constitutes a complicating factor in the other methods and must be removed, does not contribute to the amino N value. Our finding that the other N compounds of the urine, namely creatine, creatinine, uric acid, and urea, also fail to react as amino N-bearing substances in this method makes it possible to carry out this determination without previous manipulation of the urine sample. This has been found to permit the estimation of the free amino acids in the urine with a minimum expenditure of time and apparatus. The details of our experiments and application of the method to the urine are presented here.

EXPERIMENTAL

Reagents—

Cupric chloride $2\text{H}_2\text{O}$ 27.3 gm dissolved in 1 liter of water

Trisodium phosphate 64.5 gm dissolved in 1 liter of water

Borate buffer To 57.21 gm of sodium borate in 1.5 liters of water add 100 cc of N HCl and make to 2 liters with water

Copper phosphate suspension Mix 1 volume of cupric chloride solution with 2 volumes of trisodium phosphate solution and 2 volumes of borate buffer. The suspension appears to keep well in the refrigerator for about 1 week.

Thymolphthalein indicator 0.25 gm of thymolphthalein (LaMotte) dissolved in 50 cc of 95 per cent ethanol and made to 100 cc with water

* Aided by grants from the Rockefeller Foundation, Merck and Company, Inc., E. R. Squibb and Sons, and the Nutrition Foundation, Inc.

Sodium thiosulfate stock 49.6 gm (0.1 N) dissolved in 200 cc of water, add 20 cc of amyl alcohol and make to 2 liters. The amyl alcohol serves as a stabilizing agent. The 0.01 N solution is prepared from this stock solution.

Potassium iodate standard 0.3567 mg (0.01 N) of potassium iodate dried in an oven at 110° for 1 hour and dissolved in 1 liter of water. Use to standardize the thiosulfate solution.

Starch indicator Dissolve 1 gm of Lintner soluble starch in 100 cc of saturated NaCl by heating on the steam bath, cool overnight and decant the supernatant solution.

Potassium iodide Make daily in the quantity needed, 1 gm per 1 cc.
Glacial acetic acid and 1 N NaOH

Procedure

Preservation of Urine—24 hour specimens were collected in brown bottles containing 50 cc of 15 per cent HCl (by volume) and 1 cc of 10 per cent alcoholic thymol and were made to a uniform volume of 2 liters before removal of the sample for amino N determination. It has been found that under these conditions the amino N content of the specimens remains unchanged after storage for 1 week or more at room temperature.

Method—To 15 cc of urine or sample in a 50 cc volumetric flask containing at least 10 mg of amino N are added 4 drops of thymolphthalein indicator and N NaOH to the appearance of a faint green or blue color. Then 30 cc of the copper phosphate suspension are added from a graduated cylinder and the volume made to the mark with distilled water. After being mixed thoroughly by repeated inversion, the solution is allowed to stand at least 5 minutes and then filtered by gravity through folded No. 5 Whatman paper (12.5 cm) into 125 cc Erlenmeyer flasks.

The copper content of two 10 cc aliquots of this filtrate is determined iodometrically as follows. Each portion of the filtrate is acidified with 0.5 cc of glacial acetic acid and followed by the addition of 1 cc of KI solution. The solutions are titrated with standardized 0.01 N sodium thiosulfate from a 10 cc micro burette and 6 drops of starch indicator added as the end point is approached. When a low titer is encountered, the duplicate sample is titrated with 0.005 N sodium thiosulfate.

Calculations—Each cc of 0.01 N thiosulfate is equivalent to 0.28 mg of amino N. The amino N per cc of the original sample is found from the formula, cc of 0.01 N thiosulfate required for 10 cc of filtrate $\times 0.28/3$. The titration differences for duplicate 10 cc aliquots from the same sample are less than 0.02 cc. The agreements between duplicate samples containing 1 mg of amino N or more are better than 1 per cent.

Results

In order to ascertain the adaptability of the copper method to the determination of the amino N of the urine, it was clearly necessary to learn (a) the reaction of the non-amino nitrogenous constituents of the urine to the procedure and (b) the sensitivity of the method to micro quantities of the amino acids and protein digests. Data bearing on these points are presented in Table I.

For this work weighed amounts of chemically pure compounds were dissolved in water or 0.1 N HCl as necessary and the total N of the solution determined by micro-Kjeldahl analysis (6). Casein and gelatin hydroly-

TABLE I
Amino N Content of Some Nitrogen Compounds by Copper Method

Substance	Total N in sample analyzed	Amino N found	Amino N / Total N
	mg	mg	per cent
Urea	88.05	0.00	0
Uric acid	5.89	0.00	0
Creatine	1.43	0.00	0
Creatinine	9.10	0.00	0
Amigen	9.18	5.90	64.8
Casein hydrolysate	2.15	1.73	80.5
Gelatin	2.53	2.14	84.6
l(-)-Arginine HCl	19.10	4.80	25.1
l(-)-Lysine HCl	20.00	10.01	50.1
l(-)-Histidine HCl	11.60	7.55	65.0
l(-)-Histidine HCl	2.32	1.47	63.5
dl-Alanine	2.06	2.10	100.9
Glycine	4.83	4.90	101.4

sates were prepared by refluxing 10 gm. samples of commercial grades of these products with 50 cc. of constant boiling hydrochloric acid for 24 hours. The excess acid was removed by concentration *in vacuo* and the humin by filtration and the N determined by micro-Kjeldahl analysis. The amino N determinations were performed as described below on suitable aliquots of these various solutions.

It is to be noted that the normal organic nitrogenous constituents of the urine fail to react in the copper system, which indicates that the amino N values obtained by the application of the method to the urine would not suffer by their presence.

The amino N content found for the protein digest is well within the reported values. Analysis of the amino acids shows that the method is

capable of high accuracy for micro quantities of various amino acids. It appears, moreover, that glycine, alanine, arginine, and lysine yield amino N values which correspond exactly to the α -amino N content. Pope and Stevens have demonstrated this to be true not only of the other α -amino acids but also for proline and hydroxyproline. The behavior of histidine is anomalous. They have found after repeated tests that histidine yields 42.9 to 43.7 per cent of its total N as amino N instead of the normal 33.3 per cent, and interpret this to indicate the formation of the complex (histidine)₂Cu₂. We, on the other hand, have consistently found histidine to

TABLE II
Recovery of Amino N of Substances Added to Urine

Sample	Nitrogen of substances added		Total amino N in sample analyzed		Recovery of added amino N per cent
	Total mg	Amino mg	Calculated mg	Found mg	
15 cc Urine A				1.80	
Same + <i>dl</i> -phenylalanine	2.42	2.42	4.22	4.26	100.9
“ + <i>dl</i> -methionine	2.92	2.92	4.72	4.80	101.0
“ + <i>l</i> (-)-leucine	1.04	1.04	2.84	2.82	99.2
15 cc Urine B				2.19	
Same + <i>dl</i> -tryptophane	0.25	0.25	2.44	2.44	100.0
15 cc Urine C				1.22	
Same + <i>l</i> (-)-cystine	2.15	1.73	1.47	1.47	100.0
15 cc Urine D				5.44	
Same + casein hydrolysate	2.15	1.73	7.17	7.20	100.2
“ + “ “	6.45	5.19	10.73	10.60	99.1
“ + <i>l</i> (-)-arginine HCl	3.40	0.85	6.29	6.25	99.4
“ + <i>dl</i> -alanine	4.12	4.12	9.56	9.60	101.0
“ + urea	59.00	0	5.44	5.44	100.0
“ + (NH ₄) ₂ SO ₄	13.70	0	5.44	5.50	101.0

yield 63.5 to 65.0 per cent of the total N as amino N by this method on samples containing 2.32 to 11.60 mg of total N, which suggests the formation of the compound, histidine Cu.

It was further reported by Pope and Stevens that, when tryptophane, phenylalanine, methionine, and leucine were tested individually by their method, all failed to yield the expected amino N values owing to the formation of insoluble copper salts. However, they found that when these amino acids were tested in the presence of an excess of either glycine or aspartic acid the theoretical amino N values were obtained for the mixture. In our experience cystine should also be included in this category. Since an excess of amino acids does not obtain in the urine, the effect of substances present

in high concentration in the urine on the amino N values of these amino acids was studied. The addition of ammonium sulfate, ammonium chloride, and urea individually to solutions of this group of amino acids improved the amino N values somewhat, but failed to yield the calculated values. However, when these were added to the urine normal amino N values were realized, as shown in Table II. Since the quantities of these amino acids added to 15 cc of urine are far in excess of the amounts in which they might normally occur, it is evident that the amino N of these amino acids is included in the values obtained by the use of this method to the urine.

It will be further observed in Table II that the addition of urea and ammonium sulfate does not contribute appreciably to the amino N value of the urine samples, whereas casein hydrolysate, *dl*-alanine, and *l*(-)-arginine augment the amino N value as calculated.

The amino N of the 24 hour specimens of fifteen normal adult subjects on normal diets determined by this method has been found to fall between 221 and 696 mg, which corresponds to 3.25 to 4.12 per cent of the daily total N. Kirk (7) using Northrop's modification (5) of the formol method reports the daily amino N to vary from 100 to 2700 mg for normal individuals on normal fare.

DISCUSSION

It has been shown by Van Slyke and Kirk (8) that the metabolites included in the term amino N are necessarily defined by the methods used for their determination. From the available data, it appears that the character of the amino N determined by the copper method is similar to that found by the use of the formol procedure when the calculation is made from the titer between pH 7 and 9. The only differences, which are in effect self-compensating, are found in the fact that, whereas only the α -NH₂-N of histidine, both amino groups of lysine, and 0.8 of the pyrrolidine N are recorded by the formol reaction, two of the histidine nitrogens, the α -NH₂-N of lysine, and all of the pyrrolidine N are determined by the copper reaction.

Moreover, this detailed investigation of Van Slyke and Kirk has clearly indicated the superiority of the formol titration technique (4) for the estimation of amino N of the urine when compared to the Folin colorimetric method (3) and the Van Slyke gasometric procedure (2). More recently, Van Slyke and coworkers (9) have described a new gasometric method for the determination of amino acids in the urine, using the ninhydrin-CO₂ reaction which they feel offers greater specificity and convenience than any of the existing methods. It is to be noted, however, that all of the available procedures require a preliminary treatment of the urine sample for the removal of urea, ammonia, or CO₂ as the case may be. These processes are

necessarily time-consuming and in some instances may affect the over all accuracy of the determination, *e g*, the concomitant removal of the basic amino acids with ammonia by the use of permutit in the Folin technique. Our data clearly indicate that the removal of urea and ammonia from the urine sample is not necessary for the accurate estimation of the amino N by the copper method. In addition to this advantage, the fact that the determination can be accomplished without the use of special apparatus and by a single titration with a sharp end-point recommends it for routine work.

The amount of sample required for this procedure has been found to limit its use for the determination of the amino N of the blood.

SUMMARY

The copper method for the determination of the amino N has been successfully applied to the urine. The finding that the presence of ammonia and urea does not affect the amino N value permits the rapid estimation of the free amino acids in the urine.

The advantages of this procedure over the more familiar procedures are discussed.

We wish to thank Miss Jane E Frankston for some experiments performed in connection with this work.

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THE HEAT COAGULATION OF HUMAN SERUM ALBUMIN

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The present studies were designed to develop a rapid micromethod for investigation of the effects of heat on relatively concentrated solutions of human serum albumin. The method so devised is proposed for the routine study of commercial preparations, and for inquiry into some of the factors that appear to determine the thermal stability of an albumin solution. As will be clear from the paragraphs that follow, we are using the word stability in a very restricted sense: the studies pertain only to the formation of coagula or aggregates of presumably denatured albumin that are capable of producing a considerable Tyndall effect. The observed phenomenon, which will be referred to as cloud formation, is, according to the views of various authorities (1-10), the net result of two consecutive reactions: the denaturation or unfolding of the protein molecules in solution, and the flocculation, aggregation, or polymerization of the unfolded molecules to form particles that are large enough and present in sufficient concentration to scatter light and give the appearance of a cloud or turbidity. The factors that influence the rate of formation of soluble denatured protein at higher temperatures are not considered in the present study, although work is in progress on a method to ascertain the state of the protein in solution. Likewise, the formation of soluble cleavage products that might arise from thermal degradation under special conditions is not considered.

Method

The method now employed is based upon the use of thin walled capillary tubes, and the heating of albumin solutions contained therein at a constant and sufficiently high temperature until a cloud forms in the solution, that is, until the cloud point¹ is reached. Under optimum conditions the rate of cloud formation is quite rapid, and the time required for attainment of a 30 second cloud point may be recorded to within a few seconds.

Before the present studies were undertaken, Dr. Paul Tompkins of this

¹ The "cloud point" is apparently closely related to and perhaps synonymous with the coagulation point. It is so described because under the conditions of these experiments the light-scattering phase appears to the observer, at the moment of recording, as a haze or cloud rather than as a heavy coagulum.

laboratory made substantial progress with a method that called for elevation of the bath temperature at a linear rate, from an initial level of 50° or higher to the cloud point temperature, the observer recorded both the cloud point temperature and the cloud point time. Either value alone or the product of the two was found to provide descriptive information which was of qualitative interest but the data were not amenable to further analysis or interpretation.

Lepeschkin (3) has described a method that was devised upon a sufficiently small scale with respect to demand for material, but this was technically unsatisfactory for application to the problem at hand. No attempt was made to inquire, by actual trial, into its further possibilities.

Capillary Tubes—We have found that the most satisfactory capillaries are of thin walled soft glass, prepared by heating and drawing out ordinary 6 inch test-tubes, and which have about the following dimensions: length 8 cm and external diameter 1.5 mm. To indicate the thickness of wall that is considered desirable we find that ten of our tubes selected at random weigh 0.70 gm, or 70 mg each. Commercially available melting point tubes were tried but their use was discontinued owing to their insufficient length and their larger and variable diameter. The thicker walls and greater diameters of the melting point tubes do not permit a sufficiently rapid equilibration of temperature. An increase in inside diameter of from 0.7 to 1.5 mm increased the cloud point time in a given experiment from 26 to 35 seconds, i.e. by 9 seconds, and a change from one of our thin walled capillaries to a melting point tube of the same internal diameter raised the cloud point time from 33 to 36 seconds. However, melting point tubes of constant diameter could be used with fair accuracy for comparative purposes.

Filling—The protein solution is drawn about one-third of the way up the tube by gentle suction, the empty end is sealed in a small flame, and the contents are transferred to the sealed end after it cools by shaking with a whipping motion of the hand.

Water Bath—For a constant temperature bath we have found that one of brass plate (1.3 mm thick) containing three windows of plate glass is satisfactory. The internal dimensions of the bath are as follows: length 9 inches, width 6 inches, and depth 10 inches. A box of $\frac{3}{4}$ inch, five ply wood was built to fit snugly over the outside of the brass plate bath, and to furnish a moderate amount of thermal insulation. Greater insulation of the sides of the bath is in all probability unnecessary because the top of the bath is uncovered.

The diagrams in Fig. 1 illustrate the arrangement of the three windows in the bath, the position of the light projector, and the location of the temperature-regulating equipment. A concentrated beam of light from a micro projector is passed through the bath and out through a window on the

opposite side in order to avoid reflection from the bath walls. To serve as a background a piece of black bakelite, fastened to a strip of brass for support on the sides of the bath, extends down into the water about 5 inches, that is to a point just below the bottom of the windows, and thus does not interfere with the circulation of the bath water. The light that is scattered by the protein solution in the immersed capillary is observed through the front window against the bakelite background, preferably in a darkened room. Mueller (11) has reported that the intensity of scattered light is maximal at an angle of 135° to the incident beam. We have found that the cloud point is definitely more distinct when observed at an angle to the incident beam somewhat greater or less than 90° .

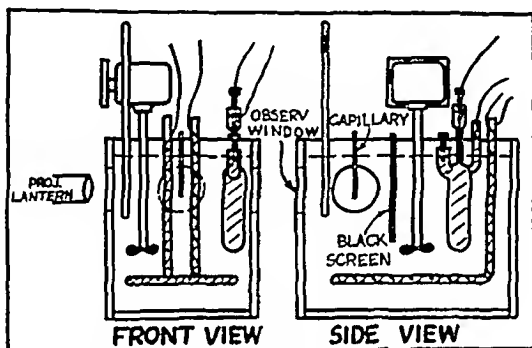


Fig 1 Constant temperature bath

The bath is refilled with distilled water every 2 or 3 days in order to keep the water clear and free from dust. The heating element employed is an 8 foot length of chromel resistance wire, wound in a $\frac{1}{2}$ inch spiral, and supported in the bath with a Pyrex rod or tubing of small diameter passed through the spiral. A mercury thermoregulator of conventional design is used, except that it is provided with a side arm into which is inserted a rubber stopper with a set screw. Rapid adjustment of the temperature is facilitated with the screw. A variation of $\pm 0.03^\circ$ in bath temperature is obtained by arranging the relay and heater circuit so that only a part of the current through the element is controlled by the relay.

Technique—In carrying out a determination it is advisable to have the solution in the capillary only half immersed, for if the filled portion is completely immersed, cloud formation will begin at the air-liquid interface and work downwards, thus decreasing the sharpness of the cloud point, and perhaps even shortening the time for its appearance. Another advantage may also be realized from partial immersion of the protein solution, if the

observer, shortly before the appearance of the cloud, lowers the capillary another 0.5 to 1.0 cm. in the bath, he will have a small amount of the unclouded solution to compare with the clouding portion, and by contrast such action will tend to sharpen the observed cloud point. The temperature of the bath should be so adjusted as to give a cloud point appearance time of 10 to 60 seconds. If the cloud point time is less than 5 seconds, we assume the value to be of doubtful significance, since an appreciable proportion of the time would probably be required for equilibration of temperature, the capillary being at room temperature prior to immersion. If the cloud point time is greater than 150 seconds, difficulties are encountered owing to loss of sharpness of the cloud point.

EXPERIMENTAL

Throughout these studies use has been made exclusively of crystalline human serum albumin or of amorphous serum albumin which, on electrophoretic analysis, appeared to contain not more than 2 per cent of globulin (mostly α -). These preparations have recently been characterized in several papers (12-16). Unless otherwise stated, 25 gm. per cent solutions of the protein were used. The solutions were prepared by mixing in the dry state albumin and sodium carbonate, the latter in quantities sufficient to give a reaction close to the desired pH. Water was added and solution of the albumin effected at room temperature or at 0°. A stock solution, 33.3 per cent albumin, was thus prepared. 75 cc. portions of these stock solutions were diluted with an appropriate volume of sodium chloride of the desired molarity. In certain of the experiments to be reported, in which other salts were studied, dilution was made with the required volume of solutions of these other substances. The final volumes after the additions mentioned were 100 cc., thus giving solutions that contained 25 per cent albumin. Suitable allowances were made for the water contained in the "dry" albumin preparations used. The investigation pertains, therefore, to 25 gm. per cent solutions of albumin, computed as for anhydrous material.

Before determinations of pH were made, the concentrated albumin solutions were diluted to 25 volumes with water. Although this procedure does not give the pH of the 25 per cent solutions actually employed, measurements were made in this way in conformity with the conventions now employed in all laboratories in which work with these solutions of human serum albumin is being done. It must therefore be emphasized that wherever reference is made in the present paper to the pH of an albumin solution we mean the pH of a solution which has been diluted to contain about 1 per cent of protein.

Determinations of the cloud point are made at several temperatures within a range so chosen as to give a series of cloud point times of from 10 to 60 seconds. The significance of this procedure will appear later.

Studies on Typical Preparations—Determinations of the cloud point time (C P) have been made thus far on thirty-six different preparations* at several different temperatures. A semilogarithmic plot of the data so obtained, $\log C P$ against temperature, gave rise to a family of straight lines such as are illustrated in Fig 2 for ten of the preparations selected at random. The slopes of these lines are very nearly the same. Later studies indicate that the slope is determined in part by the protein concentration, the nature of the added salt, and also by the pH. The linear relationship that we have observed between the logarithm of the cloud point time and the temperature is in confirmation of the findings of Bugha (17), whose

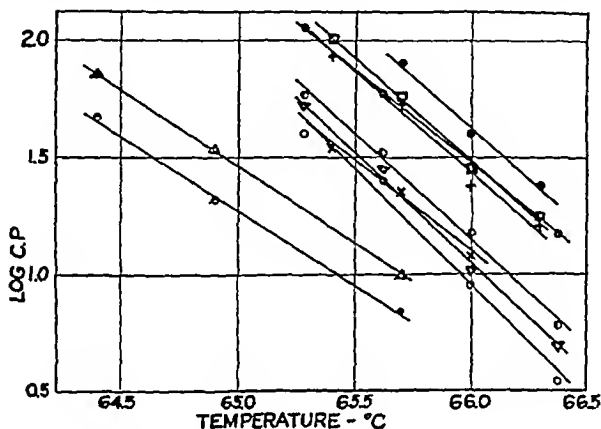


FIG 2 Cloud point-temperature relationship 1/25 dilution pH \bullet 7.15, \circ 7.01, \odot 6.81, \bullet 6.70, \bullet 6.55, Δ 7.17, ∇ 6.80, \square 6.56, \times 6.50 + 6.52

work was carried out under quite different conditions but, none the less, was such as to demonstrate the relationship mentioned.

The results obtained on thirty-six different amorphous preparations are presented in Table I. This serves to indicate the spread of values encountered in ordinary albumin processing. It also reflects the quality of the albumin, since all preparations of low cloud point are found to be poor as judged by nephelometric study in the course of standard stability tests at 57°.

In Table I it will be noted that the entries in the second and third columns

* The preparations used for this purpose were the products of an industrial house engaged in the large scale preparation of human serum albumin. As received by us, they were already in 25 per cent solution, contained 0.3 M sodium chloride, and were of pH 6.42 to 7.28, with most values clustered around pH 6.5. The sodium carbonate additions, made at the time of dissolving the albumin, were such as to add about 0.05 to the ionic strength.

are described, respectively, as "Temperature for 30 second C P" and "Calculated C P at 65°". These values were obtained in all cases by making a few determinations of C P within the appropriate temperature range. A linear plot, log C P *versus* temperature, was constructed from these observations and from this in turn the values presented in the second and third columns of Table I are readily calculated.

Quadruplicate determinations of the cloud point are made for any given temperature upon each preparation, and the average C P for the contents

TABLE I
Routine Cloud Points

Preparation No	Temperature for 30 sec C P	Calculated C P at 65	Preparation No	Temperature for 30 sec C P	Calculated C P at 65
	<i>C</i>	<i>sec</i>		<i>C</i>	<i>sec</i>
26	65 73	117	46	66 09	230
27	66 28	327	47	65 27	58
28	65 59	90	48	66 14	252
31	66 03	201	49	66 18	272
32	66 16	262	50	66 32	352
33	66 11	238	51	66 48	475
34	66 11	238	52	66 21	287
35	65 98	187	53	66 34	366
36	65 91	168	54	66 29	318
37	65 96	180	55	66 16	262
38	65 89	162	56	65 55	84
39	66 27	221	57	66 30	340
40	66 18	272	58	66 14	252
41	65 83	141	59	66 48	475
42	66 25	309	60	66 64	641
43	66 16	262	61	66 46	457
44	65 79	131	90X	64 25	7
45	66 04	204	22	65 01	31

of the four capillaries is recorded. In the 30 second range such quadruplicates should agree to ± 1 second.

It is probable that the semilogarithmic plot is linear for only a comparatively narrow temperature range. This is suggested by the observation that if the data now at hand were to be recalculated for a cloud point temperature of 50° the C P of all preparations studied of late would be many million years, a conclusion which is not in harmony with actual observations on the stability of these preparations at 50°.

Arrhenius Constant—Values for the activation energy, E , or the Arrhenius constant were calculated with the aid of the following expression,

$$\log \frac{(C P)_{t_1}}{(C P)_t} = \frac{E}{2.3R} \left(\frac{T_2 - T_1}{T_m^2} \right)$$

TABLE II
Arrhenius Constant

Effect of variations in	pH (1.25)	$-\frac{\log C/P}{t}$	T_m	$E \times 10^{-3}$	
pH, calculated from data in Fig. 3	5.1	0.29	273 + 63	150	
	5.6	0.46	+ 66	240	
	6.4	0.58	+ 67	310	
	7.9	0.55	+ 66	290	
	8.5	0.4	+ 65	210	
	9.5	0.36	+ 66	190	
	10.0	0.37	+ 66	200	
	10.7	0.18	+ 66	95	
NaCl concentration, from data in Fig. 6	NaCl				
	M				
	0	0.60	273 + 63	310	
	0.15	0.64	+ 66	340	
	0.30	0.60	+ 67	320	
	0.60	0.62	+ 69	330	
	0.90	0.74	+ 71	400	
	1.50	0.62	+ 72	340	
Protein concentration, from data in Fig. 5	Protein concentration				
	per cent				
	5	0.26	273 + 72	140	
	25	0.60	+ 67	320	
	45	0.80	+ 66	420	
Nature of added substance	0.3 M				pH
	Blank	0.60	273 + 63	310	6.73
	Chloride	0.62	+ 67	320	6.78
	Acetate	0.61	+ 68	330	6.88
	Propionate	0.80	+ 71	430	6.88
	Butyrate	0.74	+ 74	410	7.05
	Valerate	1.06	+ 79	610	6.75
	Caproate	0.56	+ 80	320	6.78
	Succinate	0.58	+ 67	310	7.03
	Fumarate	0.60	+ 68	320	6.92
	Lactate	0.69	+ 69	370	6.81
	Glucose	0.36	+ 65	190	6.75

where T_m is the absolute temperature of about the middle of the measurable cloud point range, which is seldom wider than 3° . It was assumed that $(C/P)_{t_1}/(C/P)_{t_2} \approx (k_2)/(k_1)$, where k_2 and k_1 are the reaction rate constants

for the over-all reactions which lead to the observed C/P at temperatures t and t_1 (see also (18)). For the present no attempt will be made to discuss the significance of the different values of E obtained.

Table II presents values of E with variation of pH, sodium chloride concentration, or protein concentration, and for a series of different added substances. Crystalline albumin was used to obtain the data presented in Table II.

Effect of pH—The optimum pH for the high temperature thermal stability of human serum albumin in 25 per cent solution and 0.3 M in sodium chloride was determined on two preparations. The results obtained

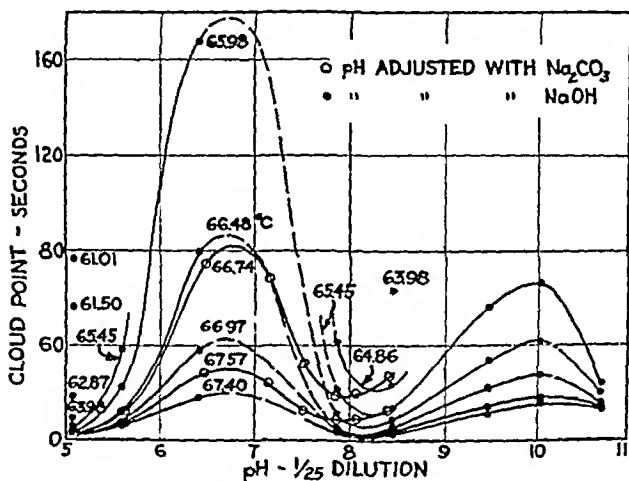


FIG. 3 Effect of pH on cloud point

on one of these preparations (crystalline) are presented graphically in Fig. 3. The appearance of a second optimum at pH 10 should be noted as well as the relationship between pH and $\Delta \log C/P / \Delta t$, this relationship is illustrated further in Fig. 4 and in Table II. The determination of the optimum pH as 6.8 was first reported by Seatchard *et al.* (13) by the use of viscometric and nephelometric methods.

Effect of Protein Concentration—An investigation was made of the effect of variations in protein concentration at constant total salt concentration (0.3 M) and constant pH, upon the cloud point temperature. The results, presented in Fig. 5, seem to indicate that the thermal stability varies inversely with the protein concentration.

Determinations of cloud point were made at several temperatures for both the 5 and 45 gm per cent albumin solutions in order to permit plot

ting $\log C P$ against temperature. The slopes of the straight lines so obtained were -0.26 and -0.80 instead of -0.63 , as was regularly obtained with this crystalline albumin preparation in 25 gm per cent solution. It appears to follow that the value of a (slope) in the equation $\log C P = at + b$ is, in part, a function of albumin concentration.

It could also be computed that this 9-fold increase in protein concentration (5 to 45 per cent) decreased the $65^\circ C P$ about 35-fold.

It is interesting to note that the increase in the slopes mentioned above in going from a concentration of 5 to 45 per cent protein corresponds to an increase in the Arrhenius constant (see Table II) of from 140,000 to 420,000 calories. However, in consideration of the decrease in $C P$ with increase in protein concentration, it would seem that the "thermal sta-

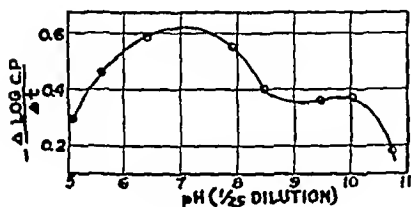


FIG 4

Fig 4 Effect of pH on $\Delta \log C P / \Delta t$

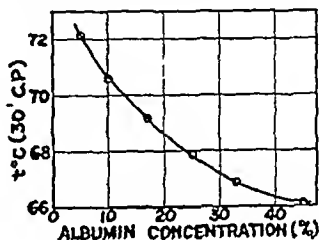


FIG 5

Fig 5 Effect of albumin concentration on cloud point

bility," as defined in this work, decreases with increase of protein concentration. It is quite probable that the reaction rate of aggregation of the denatured albumin to form light-scattering material is dependent on the concentration of the denatured albumin.

Effect of Ionic Strength—The effect of variations in the ionic strength (sodium chloride used) at constant protein concentration (25 per cent) and constant pH was also determined. These results are presented in Fig 6, they confirm essentially the findings reported by Scatchard *et al* (13). Incidentally it was observed that the slope of the $\log C P$ -temperature curve did not vary with change of sodium chloride concentration.

Effect of Constant Mole Ratio (NaCl to Protein)—Since increase of ionic strength in a system containing 25 per cent albumin is sufficient to increase the cloud point markedly (Fig 6), it was suggested that the results presented in Fig 5 might be due to the high mole ratio (NaCl to protein) that prevailed in solutions of low protein content. In consequence the experiments illustrated by Fig 7 were conducted. In these experiments a constant mole ratio for salt and protein was maintained. It is evident from

Curve II (sodium chloride) that the increase of cloud point with decrease of protein concentration (Fig 5) is not due to increase of the mole ratio (NaCl to protein) in systems of low protein content. However, the results do not permit of a very simple explanation, as is indicated by the behavior of sodium butyrate (Curve I, Fig 7)

Specific Anion Effects—In a preliminary study, replacement of part of the sodium chloride, routinely employed, with sodium acetate was investigated. With the two preparations studied there was a marked increase in the 30 second cloud point temperature. With these findings before us, a systematic study was made of other sodium salts added to a solution of

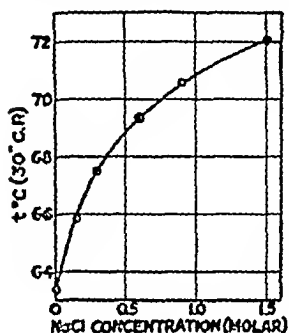


FIG 6

FIG 6 Effect of NaCl concentration on cloud point

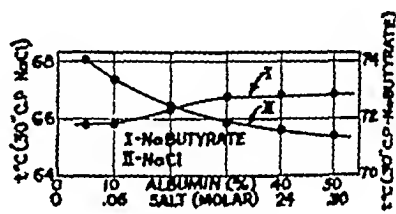


FIG 7

FIG 7 Effect on 30 second cloud point temperature of the simultaneous variation of the albumin and salt concentrations

crystalline albumin. The results are presented in Table III. The increase in cloud point with increase in length of the fatty acid anion is striking. Sodium caprylate is the highest member of the homologous series that we have studied. In 0.3 M caprylate a 25 per cent solution of serum albumin failed to show cloud formation even at 100°. It was noted, however, that the solution set to a clear translucent gel at temperatures between 80–100°.

A few experiments were performed on two routine amorphous preparations (Nos 47 and 51). The results are presented in Table IV. The absolute values for comparable experiments, reported upon in Tables III and IV, disagree because of the relatively high thermal stability of crystalline preparations. Qualitatively, however, there is good agreement.

Other Added Substances—In Table III we have included results with substances other than sodium salts. Alanine-glycine was investigated with the thought in mind that substances of high dipole moment might enhance the thermal stability. Negative results were encountered with this substance. α -Globulin (about 85 per cent α globulin on electrophoretic

analysis) was studied because it is the commonest protein impurity in serum albumin preparations. It is significant that this material did not decrease the high temperature thermal stability.

TABLE III
Increase of Thermal Stability of Serum Albumin As Affected by Added Substances

Substance added	Concentration	pH	30 sec C P temperature
	<i>M</i>		<i>C</i>
Sodium succinate	0.3	7.03	66.62
" chloride	0.3	6.78	67.29
" fumarate	0.3	6.92	68.35
" lactate	0.3	6.81	69.22
" pbenyl acetate*	0.3	6.76	78.20
" acetate	0.3	6.88	68.02
" propionate	0.3	6.88	71.48
" butyrate	0.3	7.05	74.07
" butyrate*	0.3	6.97	75.09
" valerate*	0.3	6.75	78.40
" caproate*	0.3	6.78	79.96
" caprylate*	0.3	Did not coagulate at 100°	
" chloride*	0.3	6.64	67.44
Glucose*	0.3	6.75	65.12
Sodium chloride†	0.3	6.77	67.87
Alanylglycine†	0.3	6.62	66.27
Sodium glycerophosphate†	0.3	7.49	64.68
" chloride + α -globulin†	0.3	6.91	68.05
	1.7%		
" chloride†	0.15	7.28	63.1
" "	0.3	7.06	65.4
" "	0.15	7.28	71.7
" butyrate	0.15		
" chloride	0.15	6.84	76.9
" pbenyl acetate†	0.15		
" chloride	0.15	7.15	81.5
" phenyl butyrate†	0.15		
" chloride	0.15	6.68	81.8
" caprylate†	0.15		

* Second series of experiments

† Third series of experiments

‡ Fourth series of experiments, amorphous preparation used

Nephelometry at 50° and 57°—In order to bring the present results into correlation with the routine tests applied to albumin solutions produced industrially as well as to inquire further into the effects of added salts, parallel nephelometric experiments were conducted at 50° and at 57°. To this end, bottles of appropriate shape containing 15 cc samples of test solutions of 25 per cent albumin were employed and the rate of development

of the light-scattering phase was studied. Two amorphous preparations, designated Preparations D and 94-95 respectively, were used. Readings

TABLE IV
Confirmatory Experiments with Routine Preparations

Preparation No.	Added salt (0.3 M)	30 sec C P temperature	65 C P time	Ratio of 65° C P times
		C	sec	
47	Sodium chloride	64.77	19	1
47	" propionate	65.72	115	6
47	" butyrate	67.52	2,280	120
51	" chloride	66.08	225	1
51	" propionate	67.27	2,060	9
51	" butyrate	68.76	33,650	150

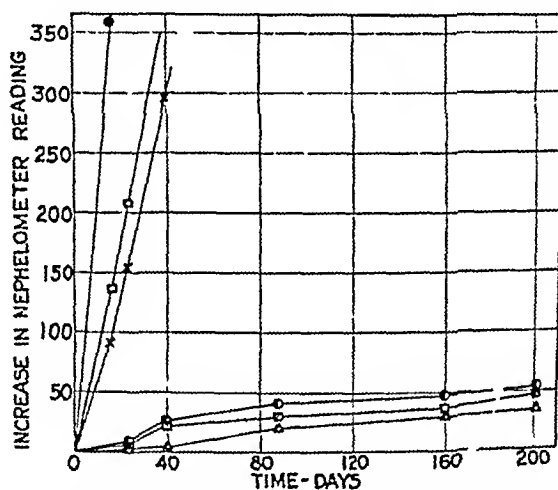


FIG. 8. Effect of various salts on the stability of Cutter Preparation D at 57°. ● 0.15 M chloride, × 0.15 M chloride and 0.15 M chloride, □ 0.15 M chloride and 0.15 M butyrate, △ 0.15 M chloride and 0.15 M phenyl acetate, ○ 0.15 M chloride and 0.15 M capronate, ◻ 0.15 M chloride and 0.15 M lactate.

were made in arbitrary units on a Zeiss nephelometer in conjunction with a Pulfrich photometer. The results are illustrated in Figs. 8 to 11.¹ The

¹ The ordinates in Figs. 8 to 11 present the increase in nephelometric readings obtained under the conditions of these experiments. An absolute reading of 20 units on the Zeiss nephelometer corresponds to a concentration of light scattering material such that a 1 inch thickness of solution is barely detectably turbid when examined in bright light with the naked eye. Solutions that read 10 units or so are completely transparent and are optically clear to the naked eye. The solutions used in these studies almost invariably have initial absolute readings of 5 to 10 units.

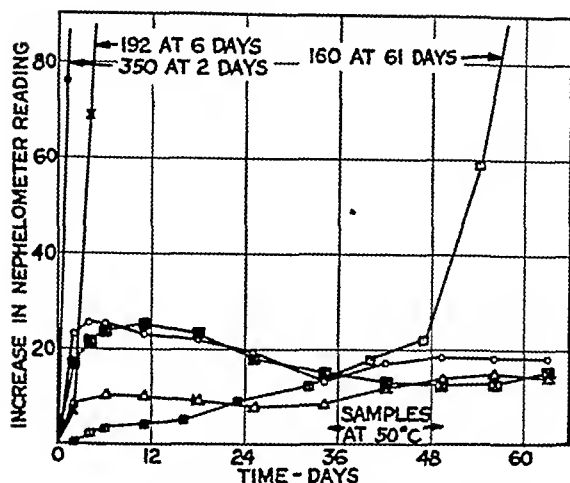


FIG 9 Effect of various salts on the stability of Preparation 94-95 at 57° ●, ×, □, △ as in Fig 8, ☒ 0.15 M chloride and 0.15 M caprylate, ○ 0.15 M chloride and 0.15 M phenyl butyrate

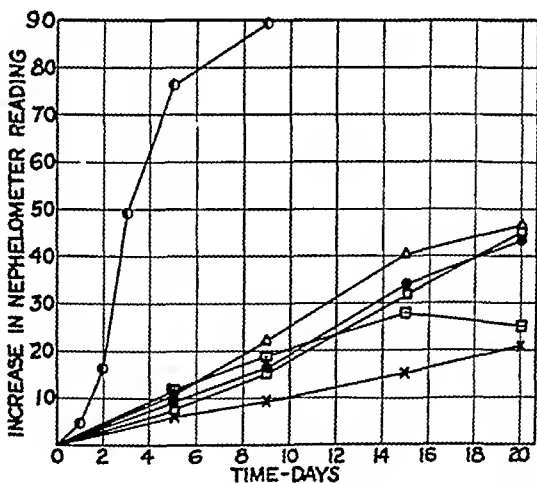


FIG 10 Effect of various salts on the stability of Cutter Preparation D at 50° The symbols have the same significance as in Fig 8

marked stabilizing effects of butyrate, caprylate, phenyl acetate, and phenyl butyrate at 57° are clearly in evidence

The instability observed in the 50° experiments with caproate and capry-

late and the comparatively high stability conferred by caprylate at 57° or at cloud point temperatures seem, on superficial examination, to be in conflict. A complete explanation cannot be presented at this time, although the data now at hand permit us to conclude that the concentration of non-polar anion, its denaturing power, the length of the carbon chain of the non-polar anion, and the temperature are the most important factors.

Preparation D, incidentally, is a singularly unstable and rather atypical preparation. It was used only to see whether we could effect stabilization of an admittedly labile product. The other albumin, Preparation 94-95, is

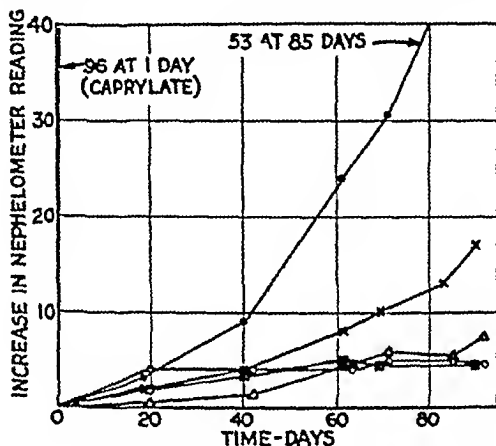


FIG. 11 Effect of various salts on the stability of Preparation 94-95 at 50°. The symbols have the same significance as in Fig. 9.

a typical representative of present day albumin preparations as produced industrially.

DISCUSSION

The most significant result of the studies reported herein is the increase in cloud point time and temperature of serum albumin solution with increase in chain length of the added fatty acid ion. From these data, however, definite conclusions may not be drawn regarding the effects of the added substances on the two reactions of the protein, denaturation and flocculation. It is presumed that the two reactions are quite separate and that the latter must be preceded by the former, that is, under the conditions of these experiments only denatured protein would be susceptible to aggregation into flocs.

Two possibilities deserve discussion if only to lay an appropriate basis

for further investigation of the phenomenon (a) The added substance may combine with the protein in its native state, giving rise to a product which is not readily susceptible to denaturation by heat and, hence, to flocculation (b) The added substance may not combine to any appreciable degree with the protein in its native state Following upon heat denaturation, however, combination may ensue with the added substance just as rapidly as opening out of the molecule exposes appropriate points of combination (These, incidentally, are assumed to be free amino groups and the aliphatic side chains of amino acid residues, especially of those that carry the free amino groups) Association with the amino groups ($R-NH_3^+$) would be through electrostatic attraction with the carboxyl group of the added substance ($R'-COO^-$) Association with the non-polar groups would be through van der Waal's forces which, considered in the aggregate, would increase with increase in length of the carbon chain of the added substance Either hypothesis necessitates the conclusion that the protein-fatty acid anion complex is of much greater solubility than denatured protein itself Not excluded from consideration is a type of combination in which association with the protein is through the non-polar portion of the added anion, the carboxyl group being immersed in the aqueous solvent

A factor that may be involved in the variable influence of the members of the homologous series is that of a varying affinity of the acid anions for the protein Steinhardt, Fugitt, and Harris (19) have reported an increasing affinity of anions for egg albumin with increasing dimensions of the anion

At present we are disposed to favor the hypothesis of combination with denatured protein, partly because the high temperature at which cloud formation ensues is, of itself, conducive to denaturation, and partly because the higher fatty acid salts (C_{12} and up) or derivatives thereof possess some degree of detergent power It is possible that a similar detergent and hence denaturing property may be manifest in some of the lower fatty acid salts such as those that we have used An increase in temperature, incidentally, appears to enhance the denaturing effect of detergents (20)

The comparatively high solubility of the protein-fatty acid anion complex, or at least its resistance to flocculation, may be attributed to a masking of all free amino groups through combination with the fatty acid anion Such a combination would leave the protein with a relatively high negative charge as conveyed by the free carboxyl groups of the protein molecule This, in turn, would militate against coalescence of particles and formation of a coagulum

Several studies are now in progress which are designed to throw further light on the data presented in this paper cloud point investigations on

heated protein-fatty acid emulsion systems before and after dialysis against 0.3 M sodium chloride, quantitative determination of denatured protein in such systems by a paper method, and electrophoretic analysis

SUMMARY

1. A "cloud point" method, in which thin walled capillaries are used as containers, is described for studies on the thermal coagulation of proteins.

2. Investigation of many preparations of human serum albumin show that, under the conditions of these experiments, a linear relationship exists between the logarithm of the cloud point time and the corresponding cloud point temperature. This relationship may be expressed by the equation $\log C/P = at + b$. The slope, a , is determined in part by the protein concentration, the pH, and by the nature of any salts present, but is independent, within limits, of the concentration of sodium chloride.

3. Cloud point data, expressed as the 30 second cloud point temperature and the 65° cloud point time, are recorded for many commercial preparations of human serum albumin.

4. A low cloud point appears to be referable to changes in the albumin itself and not to contamination with globulins or salts.

5. The optimum pH for the high temperature thermal stability of human serum albumin in 25 per cent solution and in 0.3 M NaCl was found to be 6.6, with a secondary optimum at pH 10.

6. Within the protein concentration range, 5 to 45 per cent, the cloud point was found to vary inversely with the concentration of protein.

7. In confirmation of the observations of others, the thermal stability of serum albumin was found to increase with increase of sodium chloride content.

8. In a system of constant mole ratio, NaCl to protein, the cloud point of serum albumin varies inversely as the protein concentration.

9. If the added electrolyte is a sodium salt of one of the lower fatty acids instead of sodium chloride, the cloud point increases with ascent of the homologous series, i.e., with increase in length of the carbon chain. The protective effect of phenyl acetate is as great as that of valerate, while phenyl butyrate is about as effective as caprylate. These conclusions apply to systems containing the fatty acid in the concentration of 0.15 to 0.3 M.

10. Nephelometric studies at 57° confirm, qualitatively, the results of cloud point studies in the 65–75° range. At 50° there is also qualitative agreement except in the case of caproate and caprylate.

The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

Dr. John Edsall of the Department of Physical Chemistry, Harvard Medical School, proposed the experiment with propionate, which led, in turn, to investigation of the salts of higher fatty acids. Much of the serum albumin and many other proteins required for this work were provided by the Harvard Plasma Fractionation Laboratory through the courtesy of Professor E. J. Cohn. We are indebted to the Cutter Laboratories for much cordial cooperation and to Professor Linus Pauling for assistance in interpreting some of the findings that came to light in the present study.

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B supplement which was complete for Group A, but was devoid of thiamine for Group B. A third group of animals, Group C, was given the complete vitamin supplement, but the quantity of the basal diet was limited to that which had been consumed by the rats of Group B. The effect upon fatty acid synthesis due solely to the restriction of food intake could thus be distinguished from any specific effect of thiamine deficiency in the conversion of dietary carbohydrate into fatty acids.

EXPERIMENTAL

Diet and Feeding Experiment—The diet in all the experiments comprised 10 per cent of casein (Labco, vitamin-free), 84 per cent of glucose monohydrate, 4 per cent of salt mixture (13), and 2 per cent of agar. To this was added 0.015 per cent of cod liver oil. As it proved difficult to distribute the oily supplement evenly throughout the diet, each animal was given 0.1 cc of vitamin A and D concentrate on the 5th, 12th, and 19th days, and 0.2 cc of linseed oil on the 10th day of the experiment. The daily vitamin B supplement contained 0.020 mg each of riboflavin and pyridoxine, 0.10 mg each of nicotinic acid and calcium pantothenate, and 5.0 mg of choline chloride. For Groups A and C this daily supplement was reinforced with 0.025 mg of thiamine chloride.

Each group comprised five male rats of the Sherman strain, 25 to 27 days old and weighing 32 to 46 gm. The rats were kept in individual, wire mesh bottomed cages and were weighed at frequent intervals. The weights of food and water consumed daily were noted. The basal diet was offered *ad libitum* to the animals of Groups A and B. The animals of Group C received each day the same quantity of food as had been consumed on the corresponding day by the rats of Group B. The weights of the rats and their food consumption are recorded graphically in Fig. 1.

On the 16th day of the experiment each rat was given hypodermically 0.027 cc of 99.5 per cent D₂O per gm of body weight, and, at the same time, the drinking water was replaced by 6 per cent D₂O. Water of this composition was also used to dissolve the daily vitamin supplements from the 16th day on.

Isolation of Materials—On the 21st day the animals were killed by a blow on the head, the livers were immediately removed, pooled, and frozen in solid CO₂. Glycogen was isolated essentially according to the method of Ostern and Hubl (14), by extraction of the ground organs with trichloroacetic acid and subsequent precipitation with alcohol.

The carcasses were minced and samples of body water were distilled off under reduced pressure.

After the extraction of glycogen, the residual solids from the livers were reunited with the carcasses, from which the lipids were extracted by alcohol and ether.

The extracts were taken up in petroleum ether, and the solution was washed with water and evaporated to dryness. The residue was hydrolyzed by refluxing for 2 hours with an excess of 10 per cent ethanolic KOH. The product was diluted with an equal volume of water, the non-saponifiable material was extracted with petroleum ether, washed, and finally dried *in vacuo* over P_2O_5 .

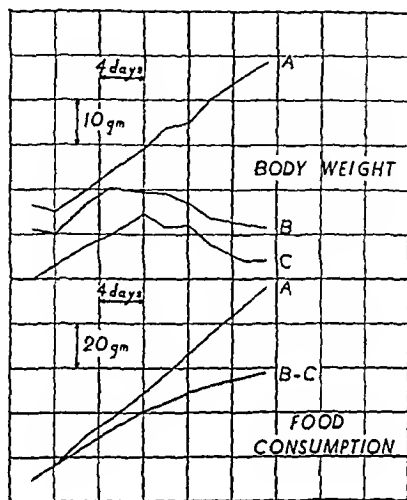


FIG 1 Three groups of young male rats have been kept on a high carbohydrate, fat free diet, with the following provisions. Group A, complete diet, offered *ad libitum*, Group B, diet lacking in thiamine, offered *ad libitum*, Group C, complete diet, restricted in quantity to the amount consumed by Group B. After 16 days, the body fluids of all the animals were enriched with D O and kept at constant level for 5 days. The body weights and the total amounts of food consumed per rat have been plotted against time.

The non-saponifiable fraction was dissolved in about 10 volumes of ether and treated at 0° with 5 volumes of a 3.2 per cent solution of bromine in acetic acid. After half an hour the cholesterol dibromide which had precipitated was filtered off, washed with acetic acid, and then with water, and dried and reconverted into cholesterol by the method of Schoenheimer (15). For deuterium analysis, the product was twice recrystallized from methanol and finally dried over P_2O_5 *in vacuo* at 100° . In each case the melting point was $146.5-147.5^\circ$.

The crude total fatty acids were liberated, taken up in petroleum ether, washed, dried, and evaporated to dryness.

Samples of saturated fatty acids were obtained from the total crude fatty

acids by precipitation of their lead salts (16, 17) The lead soaps insoluble in cold alcohol were dissolved in boiling ethanol, freed by decantation from an insoluble punt-like residue, and reprecipitated by cooling The recrystallized product was decomposed with HCl and the saturated fatty acid fraction isolated

The alcoholic solution of the soluble lead soaps was treated with H₂S, filtered, evaporated to a small volume, and diluted with water The liquid fatty acids were extracted with petroleum ether, the solution was dried over Na₂SO₄, concentrated, cooled to 0°, and treated dropwise with bromine until a brown color persisted After 18 hours in the refrigerator, the sparingly soluble tetra- and hexabromides (18) were filtered off and washed with cold petroleum ether

The petroleum ether filtrate was evaporated in a stream of N₂ and the dibromo acids dissolved in a small volume of absolute methanol This solution was refluxed with an excess of zinc dust for 2 hours, with the exclusion of moisture Thereupon 1.5 volumes of 5 N HCl in dry methanol were added and boiling continued for about 4 hours The residual zinc was removed by filtration, water was added to the filtrate, and the methyl esters of the singly unsaturated fatty acids were extracted with petroleum ether The petroleum ether solution was washed with aqueous K₂CO₃, then with water, and finally dried and evaporated to dryness From analyses of the methyl esters, the deuterium contents of the singly unsaturated fatty acids were calculated (Table I)

The methyl esters were oxidized at the position of the double bond by a modification of a method described by Armstrong and Hilditch (19) A sample was treated in dry acetone solution with 6 times its weight of finely powdered potassium permanganate which was added to the boiling solution in small portions over a period of 2 hours The mixture was then further refluxed for 18 hours, with the exclusion of moisture The acetone was distilled off and the residue suspended in water Na₂SO₄ and H₂SO₄ were added alternately to decolorize the solution and make it acid to Congo paper The mixture of acidic products and unchanged ester was carefully extracted with ether and the ethereal solution washed, first with brine and then with 20 per cent aqueous K₂CO₃ The ether layer was found to contain 15 to 20 per cent of incompletely oxidized neutral material To complete the hydrolysis of the azelaic acid monomethyl ester, 2 per cent of solid KOH was added to the K₂CO₃ solution and the mixture boiled for 1 hour On acidification, the free acids were extracted with several portions of ether After removal of the solvent, the semisolid residue was distilled in a current of steam, the volatile pelargonic and heptonic acids were extracted from the distillate with ether, and the ether evaporated The residual oil was dissolved in absolute ethanol and neutralized with saturated aqueous

Ba(OH) The barium salts, which crystallized on cooling, were collected by centrifugation, washed with a small volume of ethanol and water, and

TABLE I

Uptake of Deuterium by Body Constituents in Rats Kept on Diets Described in Text, and Supplied with D₂O in Their Body Fluids

All weights represent average values. The isotopic composition is given (1) as the actual analytical figure and (2) recalculated on the basis of 100 atom per cent deuterium in the body water.

	Group A			Group B			Group C		
	Weight	Deuterium content		Weight	Deuterium content		Weight	Deuterium content	
		(1)	(2)		(1)	(2)		(1)	(2)
	gm	atom per cent	atom per cent	gm	atom per cent	atom per cent	gm	atom per cent	atom per cent
Net weight of rat	73.4			41.4			43.2		
Dried, defatted carcass	18.4			10.0			11.4		
Body water		2.88	100.0		2.93	100.0		3.26	100.0
Total fatty acids	4.437	0.73	25.4	0.565	0.46	15.7	0.608	0.72	22.0
Saturated fatty acids		0.91	31.6		0.70	23.9			
Singly unsaturated fatty acids		0.60	20.9		0.52	17.7			
Azelaic acid		0.57	19.8		0.41	14.0			
Pelargonic, heptonic acids		0.63	21.8		0.46	15.7			
Cholesterol		0.57	19.7		0.28	9.4		0.28	8.5
Liver glycogen	0.081	0.54	18.7	0.005	1.2	41.0	0.001		
Glutamic acid from body protein		0.54	18.7		0.26	8.9			
Tyrosine from body protein		0.09	3.1		0.09	3.1			

dried at 100° over P₂O₅. The yield was about two-thirds of the anticipated amount.

Ba, theory for barium pelargonate, 30.2 per cent
 " " " " heptate, 34.1 " "
 " found, sample from Group A, 32.1 " "
 " " " " " B, 31.0 " "

The non-volatile residue from the steam distillation was recrystallized from water, with the aid of nort. The product melted at 98–101°. After one more recrystallization from water the melting point was 104° and was not depressed on admixture of an authentic sample of azelaic acid.

From the proteins of the bodies of the animals in Groups A and B glu-

tamic acid and tyrosine were isolated. The defatted tissue was hydrolyzed by boiling for 15 hours under a reflux with 20 per cent HCl. The solution was filtered, treated with norit, concentrated to a syrup, saturated with gaseous HCl at 0°, seeded with glutamic acid hydrochloride, and allowed to stand in the refrigerator for several days. The resulting precipitate was collected by centrifugation and washed with cold concentrated HCl. The product was purified by repeated solution in hot water and precipitation with HCl gas.

$C_5H_{10}O_4NCl$ Calculated, N 7.62, found (Kjeldahl), 7.60, 7.59

After removal of the glutamic acid hydrochloride, the remainder of the hydrolysate was again concentrated *in vacuo*, and most of the HCl was removed. Enough water was added to give a thin syrup and the pH adjusted to approximately 5 by the addition of NaOH and sodium acetate. The precipitate that formed was collected and treated for half an hour at room temperature with 5 per cent NaCN solution, in order to decompose any cystine, the undissolved material was centrifuged off and the sediment washed five times with 2.5 per cent NaCN solution. The supernatant and washings were combined, acidified with acetic acid, and concentrated to a small volume. The tyrosine which separated on cooling was recrystallized from water.

The deuterium contents of the materials isolated are given in Table I.

DISCUSSION

In the growth curves of the three groups of animals the effect of thiamine deficiency upon body weight becomes manifest after 7 to 10 days. The effect of thiamine deficiency upon the appetite, and consequently, upon the food consumption, appeared considerably earlier. The failure of growth and ultimate loss in weight resulting from thiamine deficiency (Group B) are almost paralleled in the animals of Group C, which received thiamine but were restricted in quantity of food to that consumed by the thiamine-deficient rats.

The bodies of the animals in Group A contained 7 to 8 times as much fatty acid as those in Groups B and C. The effect of inadequate diet on the quantity of non-fatty material was less striking. The weights of the dried, defatted tissues of the animals in Group A were less than twice those found in Groups B and C. Glycogen had almost disappeared from the livers of the animals in Groups B and C.

From the concentrations of deuterium in the body water and in the total fatty acid samples, it is possible to estimate the quantity of newly synthesized fatty acid that had been deposited during the period of D.O. administration. Rittenberg and Schoenheimer (11, 20) have shown that if animals

are maintained on a fat-free diet with their body fluids enriched with heavy water, the maximum deuterium concentration achieved in the body fatty acids is very nearly half that in the body water. This value being accepted, the calculated minimum quantities of freshly synthesized fatty acid that were deposited in each group of animals during the last 5 days of life were $2 \times 4.4 \times (0.73/2.88) = 2.25$ gm in the animals of Group A, $2 \times 0.57 \times (0.46/2.93) = 0.18$ gm in the animals of Group B, $2 \times 0.61 \times (0.72/3.26) = 0.27$ gm in the animals of Group C. These results indicate that the loss in fat, whether the result of thiamine deficiency or of restricted food intake, is referable to a very marked retardation of deposition of newly synthesized fatty acids, rather than to any excessive mobilization and catabolism of depot fat. They show, furthermore, a quantitative and qualitative similarity in the processes that resulted in loss of fat in the thiamine-deficient rats on the one hand and in the rats on restricted rations on the other hand.

The supposed specificity of the relationship of dietary thiamine to *in vivo* synthesis of fat has been questioned by other investigators. One of the lines of evidence upon which this supposed specificity is based is the fact that on a carbohydrate diet choline deprivation does not result in fatty liver unless thiamine is adequately supplied (5). However, restriction of the diet with regard to constituents other than thiamine will likewise interfere with the development of fatty liver. Thus limitation of the caloric intake to one-third of normal (21, 22), restriction of salt intake (23), and even the rigorous exclusion of lipotropic agents (24) have been shown to interfere with the development of fatty liver. Similarly, if normal growth is impeded by either thiamine deficiency or feeding of excessive nicotinamide, choline deprivation does not produce fatty liver in rats recovering from partial hepatectomy (25). Whereas the administration of thiamine to thiamine-deficient animals resulted in a marked increase in body fat, the administration of pantothenic acid plus pyridoxine to animals deficient in these fractions of the B complex provoked a very similar response (26).

For the synthesis of fat to proceed normally, an adequate nutrition must be maintained, for which a supply of thiamine is a necessary but not sufficient condition. The finding, in the present experiment, of no important analytical differences between the animals of Groups B and C strongly suggests that the loss of fat in the thiamine-deficient group (B) resulted predominantly from the loss of appetite and consequent diminished food intake and was not the immediate result of any specific effect of thiamine derivatives upon products of carbohydrate metabolism.

The isotopic composition of the fatty acids synthesized by rats in the presence and absence of dietary thiamine revealed no important differences. The saturated fatty acids were richer in deuterium than the unsaturated

acids, as has previously been shown by Schoenheimer and Rittenberg (11, 12). In their studies, the material analyzed was the total unsaturated fatty acid fraction. The fact that the more highly unsaturated, nutritionally indispensable acids acquired no deuterium, under the experimental conditions, suggested that the difference observed in isotope content of saturated and unsaturated fatty acids might be due to the presence of varying amounts of deuterium-free linoleic and linolenic acids mixed with singly unsaturated acids (20, 27). The present experiment indicates that this suggestion only partly accounts for the facts, for in this case the unsaturated acids analyzed were freed of the more highly unsaturated acids.

The difference in deuterium contents of saturated and singly unsaturated acids is taken to mean that there is a difference in the turnover rates of these substances. It is improbable that the two types of fatty acids are synthesized by unrelated routes. A likely explanation is that the primary products of fatty acid synthesis from carbohydrate precursors are the saturated fatty acids, and that oleic and palmitoleic acids are formed secondarily by dehydrogenation at the 9,10 position (17). Such an interpretation is in accord with the "hardening" effect of a high carbohydrate diet upon the body fat recently reinvestigated by Longenecker (28).

To investigate the distribution of isotope along the fatty acid chain, the singly unsaturated fatty acids were subjected to oxidative cleavage at the 9,10 position. Azelaic acid was isolated on the one hand, and a mixture of pelargonic and heptonic acids on the other. Deuterium analyses of these fragments clearly showed that the isotope was nearly equally distributed in both halves of the molecule. This had previously been reported for normal animals by Schoenheimer and Rittenberg (11). The presence or absence of thiamine did not influence this distribution.

In addition to the fatty acids, various other substances have been isolated from the bodies of the rats, and their isotopic composition determined. The differences in the quantities of cholesterol recovered from each of the three groups of animals were slight. From essentially identical quantities of non-saponifiable matter, the yields of cholesterol dibromide corresponded to 60, 60, and 80 mg. of cholesterol in the bodies of the animals of Groups A, B, and C respectively. The isotope contents of these samples, however, indicate that approximately half as much cholesterol had been synthesized per rat in the animals of Groups B and C as in the animals of Group A. As the rats of Group A weighed about twice as much as those of the other groups, the quantity of cholesterol synthesized per gm. of rat had not changed significantly in response to the varied conditions of diet.

glycogen reserves of the livers of the animals in Groups B and C were depleted at the time of death. Whereas in the sample of liver obtained from the rats of Group A about 2 out of every 10 hydrogens had arisen from the body water, in the very small sample isolated

from Group B about 4 out of every 10 had arisen in this fashion. The sample obtained from Group C was too small to permit deuterium analysis. The interpretation of these figures will be postponed until a study of the problem of glycogen turnover, now in progress, has been completed.

The glutamic acid isolated from the body proteins of the rats of Group A was about twice as rich in deuterium as that obtained from the thiamine-deficient animals. On the basis of the studies of the stability of the various H atoms in the glutamic acid molecule (29), it may be estimated that about two-thirds of all the glutamic acid present in the control animals, and about one-third of all present in the deficient animals, had been freshly synthesized in 5 days.

The carbon skeleton of tyrosine cannot be formed in the body of the rat (30) and the only known means whereby deuterium may be introduced into the molecule in the course of normal metabolism is at the α position in the process of de- and reamination (31). Both the control and the thiamine-deficient animals incorporated about one-third of the deuterium theoretically possible into their tyrosine molecules.

SUMMARY

Three groups of young male rats were kept on a high carbohydrate, fat-free diet, with the following provisions: Group A, complete diet, offered *ad libitum*, Group B, diet lacking in thiamine, offered *ad libitum*, Group C, complete diet, restricted in quantity to the amount consumed by Group B. After 16 days, the body fluids of all the animals were enriched with D₂O and kept at constant isotope level for 5 days.

In Groups B and C, the quantity of newly synthesized fatty acid deposited, calculated from the isotope values, was much less than in Group A. The findings indicate that the decrease in fat content in rats on thiamine-deficient diets results from failure of synthesis and deposition of fatty acids, and that this failure is attributable chiefly to the diminished food intake rather than to any specific action of thiamine.

The saturated fatty acids were found to be consistently richer in deuterium than the singly unsaturated fatty acids. This fact was taken to support the belief that the saturated acids are the primary products of fatty acid synthesis in rats, and that oleic and palmitoleic acids are formed from these by secondary dehydrogenation. The distribution of deuterium along the fatty acid chain was found to be approximately uniform in the fat from both Groups A and B.

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THE EFFECT OF DIETARY CHOLINE UPON THE RATE OF TURNOVER OF PHOSPHATIDE CHOLINE*

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One of the striking features of so called choline deficiency is the finding that, even though the diet is sufficiently poor in choline and its precursors to produce severe fatty liver and hemorrhagic kidney lesions in the rat, the analytical content of choline in the animal need not fall below normal levels (1, 2). From this it appears that the choline already present in the phosphatides of the animal body is not effectively utilized by the animal in the prevention of these pathological lesions. The animal seems to require a continuous supply of "new" choline, arising either from the diet or by synthesis from suitable precursors, in order to maintain a normal level of liver fat.

It was, therefore, considered of interest to determine the rate at which dietary or freshly synthesized choline was being incorporated into the body phosphatides of rats and to study the effect of the level of dietary choline upon this rate. The technique employed in this study has been to follow the appearance of N^{15} in the choline of the liver and carcass phosphatides of rats kept on a high fat diet amply supplemented with choline labeled with heavy nitrogen but otherwise poor in lipotropic factors, then, to follow the disappearance of isotope from these same fractions in the surviving animals during a subsequent period of choline deprivation.

EXPERIMENTAL

Preparation of Choline Containing N^{15} —Isotopic trimethylamine hydrochloride was prepared by the procedure of Adams and Marvel (3), and trimethylamine liberated by the addition of an excess of NaOH and swept with a stream of N_2 directly into a bomb tube containing a 10 per cent excess of ethylene chlorohydrin, cooled in dry ice (4). After all the trimethylamine had been transferred, the tube was sealed and heated for 3 hours at 85–90°. The resulting crystalline mass was dissolved in boiling absolute

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† This report is from a thesis submitted by George Ernst Boxer in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

diet, the quantity of food consumed, and the known methionine contents of casein, egg albumin, and fibrin (10), the quantity of methyl groups in the diet available for choline synthesis may be calculated if the stoichiometrical relationship of 3.2 mg of methionine to 1 mg of choline be assumed. The validity of this latter assumption is supported by the finding of Griffith and Mulford that 3 to 4 mg of methionine are biologically equivalent to 1 mg of choline (11). On this basis, the rats in the present experiment derived enough methyl groups from the diet to account for 2.3 mg of choline per day.

TABLE I

Weight and Isotopic Composition of Substances Isolated

To six groups of five rats each a dietary supplement of 50 mg of choline chloride containing 1.93 atom per cent excess N^{15} was given for 5 days. Groups of animals were killed at 3 day intervals thereafter while maintained on a basal diet devoid of choline and poor in protein. Choline has been isolated from the lipids of liver and carcass of each group of animals, and analyzed for isotope.

Duration	Weight per rat		Weight per liver	Liver lipid		Liver lipid choline	N^{15} in liver choline	Carcass lipid		Carcass lipid choline	N^{15} in carcass choline	N^{15} in total choline
	Initial	Final										
days	gm	gm	gm	gm	per cent liver weight	mg	atom per cent excess	gm	per cent body weight	mg	atom per cent excess	atom per cent excess
5	46.0	46.4	2.454	0.084	3.31	3.70	0.893	3.379	7.28	30.4	0.833	0.839
8	45.8	46.8	2.701	0.213	7.89	2.68	0.467	4.259	9.10	29.8	0.694	0.610
11	46.4	49.2	2.921	0.282	9.64	2.22	0.393	5.270	10.71	32.4	0.610	0.602
14	45.8	51.6	3.248	0.517	15.91	2.74	0.304	5.297	10.28	29.8	0.590	0.570
17	45.6	50.6	3.320	0.678	20.45	2.24	0.267	4.484	8.87	34.8	0.515	0.500
20	45.8	52.4	4.255	1.185	27.80	2.98	0.248	6.326	12.09	31.8	0.464	0.446

* The figures in this column are weighted averages of the N^{15} concentrations in the choline samples isolated from liver and carcass lipids.

During the period of choline deprivation, in addition to a striking increase in the quantity of liver lipid, an irregular increase in the quantity of depot fat was also observed. The choline contents of the lipids from the depot and the liver remained quite constant. If the high choline value of the first sample of liver lipid be excluded, the average choline content of the liver lipids is 2.6 ± 0.3 mg, a value in good agreement with that reported by Jacobi and Baumann (2). The choline content of the lipids from the remainder of the animal averages 31.5 ± 1.8 mg. This value is about 30 per cent lower than that recorded by Jacobi and Baumann. The average value of the total choline in the lipids of the whole animals during the entire period of observation is 34.4 ± 2.1 mg.

The concentrations of N^{15} in the samples of choline isolated from the lipids of the carcasses and livers of the first group of animals killed gives evidence of unusually uniform distribution of the labeled dietary molecule. The experience with amino acids labeled with N^{15} has been, in general, that when fed for a short time, the resulting isotope concentration in the liver is about 3 times as high as in the carcass. In the present experiment, the administration of labeled choline for 5 days resulted in an isotope concentration in the carcass choline very nearly as high as that in the choline of the liver. This finding indicates the rapidity with which choline is distributed to various parts of the body when it is amply supplied in the diet.

The isotope concentration achieved in the choline of the body lipids in 5 days is very nearly half of the isotope concentration in the choline fed. From the expression

$$k = \frac{1}{t} \ln \frac{z_{\max}}{z_{\max} - z}$$

a rough estimate may be made of k , that fraction of phosphatide choline arising per unit time from dietary choline during the first 5 days of the experiment, while the animals were ingesting relatively large amounts of isotopic choline. The z_{\max} represents the maximum concentration of N^{15} in the nitrogen of the body phosphatide choline obtainable on prolonged feeding of isotopic choline, and z represents the isotope concentration actually found at time t . The value for z_{\max} is assumed, in the following calculations, to be equal to the isotope concentration of the dietary choline. As this maximum could be achieved only if no source of non-isotopic choline were present, and as non-isotopic choline is synthesized to at least a small extent, the value assigned to z_{\max} is certainly greater than that which would be approached experimentally. Therefore the calculated $k = 0.114 \text{ day}^{-1}$ must be less than the true value and the half life time $t_1 = \ln 2/k = 6.1$ days, greater than the true value. This figure for the half time of the replacement of phosphatide choline by dietary choline in animals on a diet supplied with ample choline is in agreement with the corresponding value of 5.1 days, calculated from the data of Stetten (12), for adult animals on a different diet.

With the choline content of the lipids of our animals taken as constant at about 34 mg, it follows that the animals, while receiving isotopic choline in their diets, replaced approximately $34 \times 0.114 = 3.9 \text{ mg}$ per day of phosphatide choline by dietary choline containing N^{15} .

As soon as the feeding of isotopic choline was stopped, the isotope concentrations of the choline samples isolated started to drop. This decrease was more rapid in the liver than in the carcass, suggesting that the liver was an

active site for the synthesis of new, non-isotopic choline. The fraction, k , of phosphatide choline replaced by "new" choline per unit time is now given by the expression

$$k = \frac{1}{t} \ln \frac{z_0}{z}$$

where z_0 represents the isotope concentration in the lipid choline on the 5th day of the experiment and z the isotope concentration at time t thereafter. In Fig. 1, the values of $\ln(z_0/z)$ for choline from carcass and liver lipids are plotted against time.

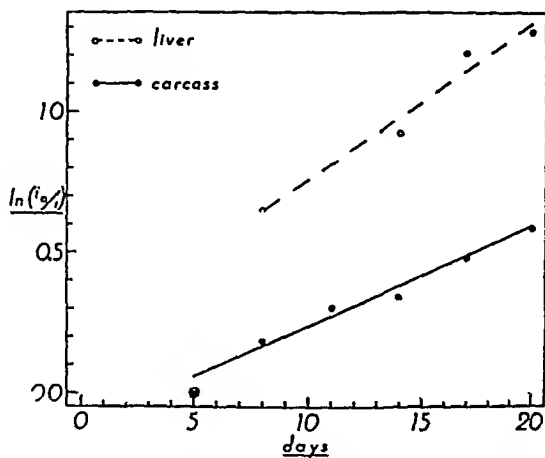


FIG. 1. To six groups of five rats each a dietary supplement of 50 mg of choline chloride containing 1.93 atom per cent excess N^{15} was given for 5 days. Groups of animals were killed at 3 day intervals thereafter, while maintained on a diet devoid of choline and poor in protein. Choline has been isolated from the lipids of liver and carcass of each group of animals, and analyzed for isotope. The isotope content of the choline on the 5th day of the experiment was designated z_0 , the isotope value on any day thereafter as z . Values of $\ln z_0/z$ are plotted against time in days. The best fitting straight line was determined by the method of least squares.

By inspection it appears that the replacement of choline in the carcass lipid proceeds at a constant rate. The same can be seen for the choline from the liver lipid for the period from the 8th to the 20th day, the same period during which the analytical amount of choline in the liver lipid stayed fairly constant. The rate of disappearance of isotope from the N of the liver lipid choline between the 5th and 8th days cannot readily be interpreted, since during this period the choline content was decreasing.

The values of k for the liver and carcass, during the period of choline

deprivation, have been estimated by determining, by the method of least squares, the slope of the straight line best fitting the experimental points plotted in Fig 1. For the carcass, the whole period has been included, for the liver, the interval between the 8th and 20th days. These figures were found for the carcass choline to be $k = 0.037 \pm 0.002 \text{ day}^{-1}$ and $t_1 = 18.9$ days, and for the liver choline, for the last 12 days, $k = 0.055 \pm 0.004 \text{ day}^{-1}$ and $t_1 = 12.5$ days. Whereas these latter figures may represent the true rate of replacement of choline in the liver phosphatide of animals on an alipotropic diet, it seems more likely that the decrease in concentration of isotope in this material has been retarded by transportation, at constant rate, of choline rich in N^{15} from the carcass to the liver.

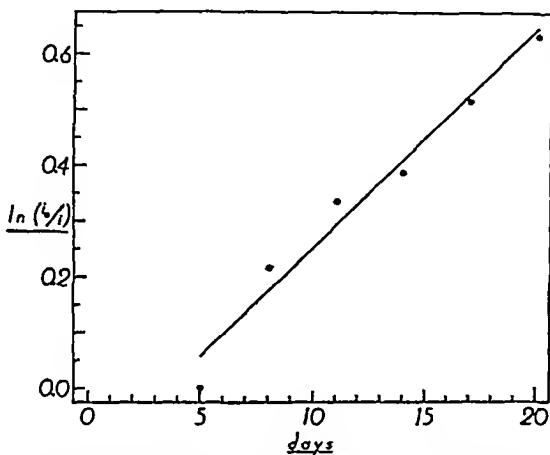


FIG 2 Weighted averages of N^{15} concentrations in total body choline have been calculated and plotted on the same coordinates as were used in Fig 1.

In order to determine the rate at which phosphatide choline is replaced by new, in this case non-isotopic, choline in the animal as a whole during choline deprivation, weighted averages of the isotope concentration in the total lipid choline have been calculated. These figures have been plotted against time with the same coordinates as previously (Fig 2). The slope of the best fitting straight line, determined by the method of least squares, was $k = 0.039 \pm 0.002 \text{ day}^{-1}$ and $t_1 = 17.7$ days. If the content of choline in the total lipids of the bodies of our rats again is taken as 34 mg, it follows that during the period of choline deprivation, $34 \times 0.039 = 1.3$ mg per rat per day of new, non-isotopic choline replaced isotopic choline previously present. The methyl groups from the methionine of the diet will amply account for this rate of choline synthesis.

It is permissible directly to compare this rate with the previously estimated rate of the replacement of phosphatide choline by dietary choline. Both figures represent the rate at which new choline, that is choline not previously a part of a phosphatide molecule, entered the phosphatides. When choline was supplied in the diet at a level of 50 mg per rat per day, the rate at which new choline entered the phosphatides was estimated as 3.9 mg per rat per day. When no supplementary choline was given, this rate dropped to a value of 1.3 mg.

Since fatty livers developed in our animals while the total quantity of choline present in the phosphatides remained constant, and since when choline was supplied in the diet a 3-fold increase was noted in the rate of incorporation of choline into the body phosphatides, it is proposed that the development of fatty liver is intimately connected with the rate at which the choline phosphatides are turned over in the body while their quantity remains constant. This view is in accord with the findings of Perlman and Chaikoff (13) who have demonstrated, under experimental conditions different from ours, that the administration of choline to rats markedly accelerates the rate of turnover of the phosphorus in the phosphatides.

From the points in Figs. 1 and 2 there is no evidence of any change in the mechanism of choline metabolism at about the 7th day, which could account for the regression of renal disease noted by Griffith and Wade (14, 15). It is perhaps worth noting that in their experiments 1 to 2 mg of choline sufficed to prevent the renal damage, which is approximately what the rats in the present experiment were able to synthesize. It is of interest also that the results of Jacobi, Baumann, and Meek (1) show a synthesis of at least 1 mg of choline per day in rats maintained on a choline free diet.

SUMMARY

With the aid of isotopic (N^{15}) choline, the rate at which this constituent of the phosphatides is replaced in rats has been determined, both on an adequate and on an alipotropic diet. When choline was fed, the half life of phosphatide choline was about 6 days, and the daily replacements of choline in the phosphatides, 3.9 mg per rat. When no choline was fed, while the rats were developing severe fatty livers, the half life of choline increased to 18 days, and the daily replacement decreased to 1.3 mg. The effect of choline deprivation has been markedly to retard the rate of incorporation of new choline into the phosphatides of the body without altering the quantity of choline in the phosphatides. It is proposed that the appearance of fatty liver is referable to this change in the rate at which dietary or freshly synthesized choline enters the body phosphatides.

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THE PEPTIDASES OF INTESTINAL MUCOSA

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Of the many and diverse peptidase activities present in intestinal mucosa, the most frequently studied were the enzymes hydrolyzing leucylglycine and leucylglycylglycine. Earlier investigators of the enzymatic splitting of leucylglycine, alanyl-glycine, and glycylglycine derived from their observations the impression that a single dipeptidase was being studied. Linderström-Lang (1), however, demonstrated that the two dipeptides, leucylglycine and alanyl-glycine, were split by distinct enzymes which could be differentiated on the basis of different stability and pH optima. One of these enzymes, designated leucylpeptidase, was assumed to hydrolyze leucylglycylglycine and leucyl-diglycylglycine as well as leucylglycine. Thus, the older theories of peptidase specificity relating to a dipeptidase, an aminopeptidase, and an occasional carboxypeptidase as the sole components of the intestinal crepsin were shown to be erroneous. Since then evidence has been brought forward indicating the existence of additional types of enzymes in intestinal mucosa such as prolinase (2) and prohidase (3).

Most of these earlier results were obtained by the use of enzymes, which were purified by the adsorption-elution technique and with the help of all too few substrates. More recently it was discovered that the activity of various peptidase preparations obtained from glycerol extracts of intestinal mucosa by precipitation with acetone was decreased on dialysis or absorption and could be partially or totally restored by the addition of various metal ions (4). Moreover, it was demonstrated that the restoration of the activity towards leucylglycine, as produced by the combination of the activating metal ion with the inactivated peptidase, is a time reaction (5). Thus, one of the intestinal peptidases was shown to be a conjugated protein, although in a sense somewhat different from Willstätter's frequently cited theory which postulated that each enzyme consists of a colloidal carrier and an active group regarded as responsible for the enzymatic specificity. It is shown below that prohidase is also a manganese protein compound.

The experiments reported in this communication were undertaken with the intention to differentiate, characterize, and purify several of the peptidases present in intestinal mucosa. Because of the present war emergency, this work had to be terminated before it had attained its goal.

l-Leucine-aminoexopeptidase

The enzyme discussed in this section corresponds to Linderström-Lang's leucylpeptidase

Preparation and Specificity—The activity of leucine-aminoexopeptidase may be easily differentiated from most of the other activities present in crude aqueous extracts of mucosa by a precipitation with acetone followed by a fractionation with ammonium sulfate

345 gm of mucosa from twenty-five 2 to 3 foot lengths of the upper end of hog duodenum were ground with 35 gm of sand and 350 cc of water. The extract was centrifuged and the mucosa reextracted with 350 cc of water. The combined yellowish opalescent extracts (Preparation A) consisted of 650 cc and contained 3.98 mg of protein N per cc. The proteolytic coefficient for *l*-leucylglycine, C_{LG} , was 0.062. Preparation A represented 80,500 enzyme units, as defined earlier (6), corresponding to 31 leucine-aminoexopeptidase units per mg of protein N.

The precipitate obtained by mixing chilled Preparation A with an equal volume of cold acetone was collected and thoroughly washed with acetone.¹ The dried powder (16.8 gm) was twice extracted with water at 40°, and the solution clarified by filtration with Celite. This solution (Preparation B) consisted of 360 cc, and contained 1.30 mg of protein N per cc. C_{LG} was 0.35. Preparation B contained 82,000 enzyme units, corresponding to 175 units per mg of protein N.

To 350 cc of Preparation B were added 84.7 gm of ammonium sulfate to bring about a 40 per cent saturation. The precipitate was discarded and to the clear solution (Celite filtration) were added 48 gm of ammonium sulfate to give a 60 per cent saturation. The precipitate was collected and dissolved in water. After dialysis in the cold against distilled water, the inactive precipitate was filtered off and discarded. 66 cc of a clear, faintly yellowish solution (Preparation C) were obtained. It contained 0.360 mg of protein N per cc. C_{LG} was 2.1. Preparation C corresponded to 25,000 enzyme units and contained 1050 units per mg of protein N. A 34-fold purification was thus obtained and a yield of 31 per cent of the original activity of Preparation A. The purified preparation was stable for many weeks when kept in the cold under toluene. A lyophilized preparation kept for several months in the dry state in the cold had not lost any of its activity when redissolved and tested in the presence of Mn^{++} .

Table I indicates that the bulk of the activities present in the crude mucosa extract, Preparation A, was removed in the course of further purification. Several conclusions may be drawn from the data presented in

¹ The crude, dry solid may be kept in the desiccator for months with no apparent loss of activity.

Table I For example, 99 per cent of the activities towards *l*-alanylglycine and glycyglycine has been removed and, therefore, must be due to an enzyme or enzymes distinct from leucine-aminoexopeptidase. However, the small residual activities towards these substrates may or may not be due to the leucine-aminoexopeptidase. It will be noted that the activity towards *d*-leucylglycine has been reduced from one-eighty-second in Preparation A to less than 0.00005 in Preparation C of the activity towards the *l* isomer.

TABLE I

Activities of Crude and of Purified l-Leucine aminoexopeptidase Preparations towards Various Substrates

For the crude extract (Preparation A), no activator was added for the tests, since no activation was detectable. For the other tests, the enzyme was incubated with 0.02 M MnSO_4 buffered at pH 8.0 at 40° for 3 to 4 hours. A test aliquot was then added to the buffered peptide solution.

Substrate	Proteolytic coefficient C			Yield per cent
	Preparation A	Preparation B	Preparation C	
<i>l</i> -Leucylglycine	0.062	0.35	2.1	31
<i>d</i> -Leucylglycine	0.00076	0.0010	<0.0001	
<i>l</i> -Leucylglycylglycine	0.11†	0.55†	2.2	18
<i>l</i> -Leucinamide	0.022		1.0	42
Glycyglycine	0.025		0.0065	0.24
Benzoyl- <i>l</i> -argininamide	0.0005		<0.0001	
Glycyl- <i>l</i> -leucine	0.034		0.012	0.32
<i>l</i> -Alanylglycine	0.26	0.042	0.17	0.60
Glycyl- <i>l</i> -proline	0.015		0.035	2.1
<i>l</i> -Prolylglycine	0.035		0.027	0.71
Glycyl- <i>l</i> -alanine			0.021	
<i>l</i> -Leucyl- <i>l</i> -leucylglycine			1.6	
Benzoyl- <i>l</i> -leucylglycine			<0.0001	

* Yields are given as $\frac{100 \times \text{total activity of Preparation C}}{\text{total activity of Preparation A}}$

† Initial C values

This appears to be conclusive evidence that distinct enzymes cause the splitting of the two stereoisomeric peptides.

The kinetics of *l*-leucine-aminoexopeptidase are complicated by the fact that the enzyme, in the course of purification, becomes increasingly responsive to activation by Mn^{++} or Mg^{++} . Moreover, when samples of the purified enzyme are incubated at pH 7.8 to 8.0 with Mn^{++} ions, before the substrate is added, the enzymatic activity increases during the first few hours. Longer incubation, however, produces a considerable decrease of

enzymatic activity and after an incubation period of 24 hours about 95 per cent of the activity is lost (Table II)

This behavior is the same whether the substrate is *l*-leucylglycine, *l*-leucylglycylglycine, or *l*-leucinamide. The fact that both the reaction of Mn^{++} with enzyme and the subsequent inactivation run approximately parallel for the three substrates suggests that all three substrates are hydrolyzed by the same enzyme.

The enzyme which is responsible for the activity of Preparation C towards *l*-leucylglycine cannot be called a dipeptidase, since it splits *l*-leucylglycylglycine, *l*-leucyl-*l*-leucylglycine, and *l*-leucinamide as fast as *l*-leucylglycine. The splitting of leucinamide indicates that the enzyme does not require a

TABLE II

Effect of Incubation of l-Leucine-aminoexopeptidase with $MnSO_4$ upon Its Activity towards Several Substrates

The enzyme (protein N = 36 γ per cc) was incubated at 40° and pH 8.0 with 0.02 M $MnSO_4$ before the respective substrate was added. The hydrolyses were carried out at 40° and pH 7.8 to 8.0. Protein nitrogen for the 3 hour test was 2.88 γ per cc, for the 25 hour test, 5.76 γ per cc.

Time of preceding incubation	Action on <i>l</i> -leucylglycine			Action on <i>l</i> -leucylglycylglycine			Action on <i>l</i> -leucinamide		
	Time	Hydrolysis	C	Time	Hydrolysis	C	Time	Hydrolysis	C
hrs	min	per cent		min	per cent		min	per cent	
3	30	33	2.0	30	32	1.9	30	20	1.12
	60	59	2.2	45	48	2.2	60	31	0.94
	75	64	2.1	60	60	2.3	120	55	1.01
							150	64	1.03
25	180	25	0.12	180	28	0.14	150	18	0.100
	300	35	0.11	300	44	0.15	180	21	0.100
	330	39	0.11	330	46	0.14	300	29	0.087
							330	32	0.088

free carboxyl group. A free amino group seems to be necessary for the action of this enzyme, since no splitting of benzoyl-*l*-leucylglycine could be observed. The data at present available make it therefore appear that the enzyme should be classified as *l*-leucine-aminoexopeptidase, in accordance with a previous suggestion (7, 8). *l*-Leucine aminoexopeptidases have previously been found to be present in extracts of beef spleen, beef kidney, and swine kidney. The kinetics of the intestinal *l*-leucine aminoexopeptidase were studied extensively with the expectation that it might become possible to establish on a quantitative basis the homospecificity (Bergmann (9) p. 49) of the leucine-aminoexopeptidases from intestinal mucosa, on the one hand, and from spleen and kidney, on the other. However, the instability

of the manganese-activated intestinal enzyme mentioned above has hitherto frustrated all such efforts

Berger and Johnson (10) have reported that the addition of cysteine enhances the effect of Mn^{++} . We have found that our leucine-aminopeptidase is partially inactivated when incubated with cysteine before Mn^{++} and substrate are added. Table III shows the result of such an experiment. The first order velocity constants are calculated in order to illustrate the changes which take place. Control Experiment A shows that incubation

TABLE III

Effect of Incubation of Protein of l-Leucine aminoxoepitidase with Cysteine and with $MnSO_4$

Incubations were carried out at 40° and pH 7.9. The test substrate was *dl*-leucylglycine. Protein N = 1.97 γ per cc. in the test solution and 24.7 γ per cc. in the incubation mixture.

Experiment A Control incubation of enzyme in absence of $MnSO_4$ test solution contained 0.01 M $MnSO_4$		Experiment B Incubation of enzyme with 0.01 M cysteine in absence of $MnSO_4$ test solution contained 0.01 M $MnSO_4$		Experiment C Incubation of enzyme with 0.01 M $MnSO_4$ test solution contained 0.0008 M $MnSO_4$ introduced with protein	
Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis
Immediate test of incubation mixture before incubation					
min	per cent	min	per cent	min	per cent
60	18	60	18	60	12
90	26	90	27	90	21
180	71	180	68	180	60
240	83	240	82	240	78
360	92	360	91	360	87
Test after 3 hrs incubation					
60	19	60	2	60	33
120	38	120	10	120	67
180	70	180	19	150	82
210	80	210	24	180	88
				210	92

of the inactive protein alone for 3 hours at 40° effects no appreciable change. The instantaneous addition of cysteine in Experiment B gives results identical with the control. However, after 3 hours, a large inactivation is shown. Experiment C shows the results of incubating Mn^{++} and enzyme. Initially, the test hydrolysis shows a slightly lower rate which is the result of the somewhat lower Mn^{++} concentration in the test solution. After 3 hours, the initial splitting of the substrate is considerably higher.

Leucine-aminoxoepitidase is activated by Mg^{++} as well as Mn^{++} .

However, higher concentrations of Mg^{++} are necessary and, in addition, still longer times are required for maximal activation. As with the Mn^{++} activations, the data run in parallel manner for the three different substrates when tested with the Mg^{++} enzyme.

Leucylglycine was introduced about 15 years ago as a substrate for the characterization of ereptic dipeptidase. The fact is now sufficiently established that the intestinal "leucylpeptidase" responsible for the manganese-activatable hydrolysis of *l*-leucylglycine and *l*-leucylglycylglycine can be neither a dipeptidase nor a carboxypeptidase but must be an aminoxopeptidase of a specificity adapted to the side chain and configuration of *l*-leucine. It is to be expected that intestinal mucosa contains other aminoxopeptidases² of different side chain and antipodal specificities and that each of these aminoxopeptidases also acts upon dipeptides that fulfill the side chain and antipodal requirements of the enzyme. Consequently, the occurrence in intestinal mucosa and elsewhere of dipeptidases which are specifically and exclusively adapted to the hydrolysis of dipeptides must be regarded as doubtful until new evidence for their existence is brought forward.

Prolidase

In 1937, it was reported (3) that glycyl-*l*-proline is split by an enzyme distinct from the previously known peptidases. This enzyme was named "prolidase." It was shown that prolidase is only slightly inhibited by cyanide in concentrations which strongly inhibit the splitting of *l*-leucylglycine and *l*-alanylglycylglycine. In contrast to other proteolytic enzymes, prolidase is capable of hydrolyzing a peptide bond that does not contain peptide hydrogen. In order to facilitate further studies of prolidase, an attempt was made to improve its separation from other enzymes of the intestinal mucosa.

Prolidase remains in the mother liquor when *l*-leucine-aminoxopeptidase is prepared from a crude aqueous extract of intestinal mucosa by precipitation with acetone. The mother liquor was lyophilized. The material thus obtained was extracted several times with ether and the residue after drying *in vacuo* dissolved in water. The aqueous solution was dialyzed and subsequently tested for its activity towards various substrates. The findings reported in Table IV indicate that the predominant activity was found to be prolidase.

The enzymatic activities present in the crude mucosa extract towards glycylglycine, *l*-prolylglycine, and glycyl-*l*-leucine are destroyed by the

* Some of these will be components of the enzyme fraction usually designated intestinal aminopolypeptidase, some may occur in other enzyme fractions obtainable from intestinal mucosa.

acetone treatment and are not found either in the acetone precipitate or in the mother liquor and, therefore, cannot be identical with prolidase. The residual activity towards glycyl-*l*-alanine, which accompanies the acetone-purified prolidase, is apparently distinct from the extremely high activity towards *l*-alanylglycine present in the crude mucosa extract. That glycyl-*l*-proline (GP) and glycyl-*l*-alanine (GA) are split by different enzymes is indicated by the varying values of the quotient C_{GP}/C_{GA} obtained by different methods of treatment, *e.g.*, the acetone-purified prolidase shows $C_{GP}/C_{GA} = 10.3$, while a lead acetate-treated prolidase (see below) gives $C_{GP}/C_{GA} = 29.4$. The data of Table IV show that prolidase

TABLE IV

Activities Present in Crude Prolidase Preparations (Acetone Method)

Hydrolyses were carried out at 40° and pH 7.8 to 8.0. Enzyme concentration, 0.165 mg of protein N per cc of test solution.

Substrate	Hydrolysis		
	No Mn ⁺⁺ added to test solution	Test solution contained 0.001 M MnSO ₄	
	26 hrs	2 hrs	26 hrs
	per cent	per cent	per cent
<i>l</i> -Alanylglycine	1	1	8
Glycylglycine	-1	-1	3
<i>l</i> -Leucylglycine	-1	1	12
<i>l</i> -Leucylglycylglycine	1	1	9
<i>l</i> -Prolylglycine	0	-1	4
Carbobenzoxylglycyl- <i>l</i> -proline	0		0
Glycyl- <i>l</i> -leucine	0		0
Glycyl- <i>l</i> -phenylalanine	0	0	2
Glycyl- <i>l</i> -alanine		13	59
Glycyl- <i>l</i> -proline	47 (2 hrs)	78	

is a highly specific enzyme with little or no action on peptide bonds containing a peptide hydrogen, as already suggested by Bergmann and Fruton (3).

These data also show that prolidase is activated by Mn⁺⁺ and may be added to the list of metal-activated peptidases. Unlike leucine-aminopeptidase, which is activated by both Mn⁺⁺ and Mg⁺⁺, prolidase is not activated by Mg⁺⁺. Other metal ions tested, such as Co⁺⁺, Cu⁺⁺, and Zn⁺⁺, strongly inhibited the enzyme. For the splitting of glycyl-*l*-proline by prolidase in the presence of Mn⁺⁺ satisfactory pseudo first order reaction velocity constants are obtained (Table V). This behavior may be used to advantage in future attempts to define the specificity of prolidase in terms of the classification of proteolytic enzymes suggested previously (8).

Because only small quantities of enzyme were available from the acetone-

containing mother liquor, another method of preparing prolidase was developed. This latter method possesses the advantage that larger quantities of much more active material can be easily prepared. This material simultaneously contains large amounts of a second enzyme which hitherto was regarded as an aminopolypeptidase (Johnson and Berger (9))

TABLE V
Kinetics of Prolidase Action on Glycyl-L-Proline

The enzyme was prepared by the acetone method. Hydrolyses were carried out at 40° and pH 8.0. The test solutions contained 0.01 M MnSO_4 .

Protein N per cc test solution	Time	Hydrolysis	C_{GP}	C_{GP} average
mg	min	per cent		
0.149	30	27	0.031	0.034
	60	49	0.033	
	75	57	0.033	
	90	68	0.037	
	120	78	0.037	
	150	81	0.032	
0.0992	30	23	0.038	0.035
	60	35	0.032	
	75	42	0.032	
	90	51	0.035	
	120	64	0.037	
	150	72	0.037	
0.0496	30	13	0.041	0.037
	60	22	0.036	
	90	30	0.035	
	120	39	0.036	
	150	47	0.037	
	180	55	0.039	
0.0198	60	9	0.035	0.036
	120	18	0.036	
	150	21	0.035	
	180	26	0.036	
0.149 (No MnSO_4 added)	30	17	0.018	0.018
	45	25	0.018	
	60	34	0.020	
	90	42	0.018	
	120	47	0.015	

p. 69, (11)) The specificity of this enzyme will be discussed in the following section.

An aqueous extract of hog intestinal mucosa is prepared as described above for leucine-aminoexopeptidase. With an aliquot of this crude extract, the amount of a saturated lead acetate solution necessary for maxi-

mal precipitation is determined. The calculated quantity is then added to the bulk of the solution and the precipitate removed by centrifuging. To the supernatant solution one adds an amount of disodium phosphate in excess of that necessary for the removal of the lead. After 30 minutes, the precipitate is removed by filtration with Celite and the clear filtrate dialyzed overnight against distilled water in the cold. Solid ammonium sulfate is then added to 40 per cent saturation, and the slight precipitate is discarded. Additional ammonium sulfate is added to bring to 60 per cent saturation and the precipitate is collected. The precipitate is dissolved in water, filtered clear, and dialyzed against distilled water until free of sulfate ions. This solution, in the presence of 0.001 M Mn^{++} , has a prolidase activity, C_{GP} , of 0.47, while the crude extract had a C_{GP} of about 0.015. Thus, the purified prolidase contained about 30 times as much activatable prolidase per mg of protein N as the crude extract. The purified prolidase has, in the absence of added Mn^{++} , a C_{GP} of 0.075. Thus, the increase in activity produced by Mn^{++} is greater than 6-fold. With other preparations, the increase was even greater, in one case, more than 8 times.

The crude extract and the purified solution also cause a splitting of glycyl-L-hydroxyproline. This hydrolysis is also activated by Mn^{++} ions (in one instance, as high as 16 times). The splitting of the proline compound (GP) by the activated enzyme is about 8 times more rapid than that of the hydroxyproline compound (GHP). The proteolytic quotient C_{GP}/C_{GHP} is practically identical in the presence of Mn^{++} for the various enzyme preparations presented in Table VI. It will be noted, however, that the proteolytic quotient of the purified prolidase, Preparations 8 and 9 in Table VI, is higher in the absence of added manganese.

These data can be interpreted in two ways. Either we are dealing with a single enzyme which shows different affinities for the substrates at different manganese concentrations or the two substrates are hydrolyzed by two extremely similar enzymes both of which are activated by Mn^{++} and are affected in exactly the same manner by several purification procedures: acetone, lead acetate, ammonium sulfate, and inactivation at 60° . The latter assumption is highly improbable. It appears, therefore, most likely that the first hypothesis is the correct one, since the ratio C_{GP}/C_{GHP} after maximal activation is the same as for the crude extract when the addition of Mn^{++} has no effect.

In Table VII are presented some experiments with various proline and hydroxyproline compounds. It is apparent that as compared with glycyl-L-proline and glycyl-L-hydroxyproline, the amino- and carboxyl-substituted compounds are split rather slowly. In fact, the slight activity towards these latter substrates may be due to different enzymes. In particular, the hydrolysis of carbobenzoxyglycyl-L-prolinamide and carbobenzoxyglycyl-L-

hydroxyprolinamide cannot be ascribed to prohidase or prolinase, but must be attributed to one or several endopeptidases. No splitting was observed of the two diketopiperazines.

l-Prolylglycine and *l*-hydroxyprolylglycine were hydrolyzed very slowly by the purified extract. Comparison with the experiments reported in the lower section of Table VII shows that these two substrates are split more rapidly than the respective reciprocal peptides by the crude extract. Apparently, the major part of the activity towards prolylglycine and hydroxyprolylglycine has been removed during the purification of the extract.

TABLE VI

Proteolytic Quotients for Glycyl-l-Proline and Glycyl-l-Hydroxyproline

The hydrolyses were carried out at pH 8.0 and 40°. Preparations 1 to 7 contained 0.001 M MnSO_4 in test solutions.

Preparation	C_{GP}	C_{GHP}	C_{GP}/C_{GHP}
1 Crude extract of mucosa	0.012	0.0015	8.0
2 " " " "	0.015	0.0019	7.9
3 Lead acetate-purified <i>l</i> -prohidase	0.29	0.034	8.5
4 " " " " from Preparation 2	0.47	0.065	7.2
5 Preparation 4 heated to 60° for 10 min, 90% of activity destroyed	0.0036*	0.00047*	7.7
6 <i>l</i> -Leucine aminoxo-peptidase	0.028	0.0034	8.2
7 Preparation 6 reprecipitated with 50% acetone and dialyzed	0.027	0.0035	7.7
Average			7.9
8 Preparation 3, no added Mn^{++}	0.039	0.0029	13.4
9 " 4, " " "	0.075	0.0040	18.8

* These are *K* values, since no nitrogen determinations were performed on the heated solution.

Of particular interest is the relatively rapid hydrolysis by the crude extract of *l*-hydroxyprolylglycine, a substrate not previously studied.

Other Enzymes

The preparation of prohidase by the lead acetate procedure described in the preceding section is a rich reservoir of other enzymatic activities responsible for a rapid splitting of tripeptides such as *l*-leucylglycylglycine, *l*-alanylglycylglycine, diglycylglycine, diglycyl-*l*-proline, and diglycyl-*l*-hydroxyproline. There is no activation by Mn^{++} ions and in some cases a definite inhibition.

The presence of an enzyme which splits *l*-leucylglycylglycine is of special interest. This enzyme was found to act upon *l*-leucylglycylglycine at the peptide bond adjacent to the free amino group with the formation of leucine and glycylglycine. This enzyme cannot be identical with *l*-leucine-aminoexopeptidase. This conclusion is derived not only from the already mentioned fact that this enzyme is not activated by manganese, but also from

TABLE VII

Hydrolysis of Proline and Hydroxyproline Compounds by Extract of Intestinal Mucosa, Purified by Lead Acetate

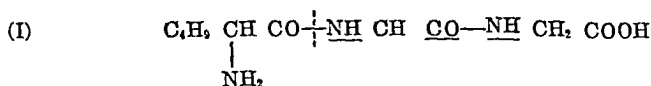
All substrates were present in a concentration of 0.05 mM per cc. at 40°, buffered at pH 8.0 with 0.02 M veronal. The test solutions contained 0.001 M MnSO₄. Lead acetate-purified prolidase was employed as the enzyme.

Substrate	Enzyme concentration mg. protein V per cc.	Hydrolysis	
		1½ hrs	24 hrs
		per cent	per cent
Glycyl- <i>l</i> -proline	0.00875	40	99
Glycyl- <i>l</i> -hydroxyproline	0.109	55	100
<i>l</i> -Prolylglycine	0.219	1	20
<i>l</i> -Hydroxyprolylglycine	0.219	0	12
Glycyl- <i>l</i> -proline diketopiperazine	0.219	0	0
Glycyl- <i>l</i> -hydroxyproline diketopiperazine	0.219	0	0
<i>l</i> -Prolinamide	0.107	1	3
<i>l</i> -Hydroxyprolinamide	0.107	-1	7
Carbobenzoxyglycyl- <i>l</i> -proline	0.320	3	22
Carbobenzoxyglycyl- <i>l</i> -hydroxyproline	0.320	1	9
Carbobenzoxyglycyl- <i>l</i> -prolinamide	0.320	5	27
Carbobenzoxyglycyl- <i>l</i> -hydroxyprolinamide	0.320	2	16
Carbobenzoxy- <i>l</i> -prolyl- <i>l</i> -proline	0.219	0	2
Glycyl- <i>l</i> -proline*	0.198	47	
Glycyl- <i>l</i> -hydroxyproline*	0.494	38	
<i>l</i> -Prolylglycine*	0.198	56	
<i>l</i> -Hydroxyprolylglycine*	0.494	59	

* These experiments were performed with a crude aqueous extract of intestinal mucosa as the enzyme, no Mn⁺⁺ was added.

our observation that the preparation discussed here splits *l*-leucylglycylglycine about 80 times as fast as *l*-leucinamide and 500 times as fast as *l*-leucylglycine. If this enzyme were homospecific with the manganese-activated *l*-leucine-aminoexopeptidases, it should split the three substrates, *l*-leucylglycylglycine, *l*-leucylglycine, and *l*-leucinamide, with proteolytic coefficients of the same order of magnitude. The possibility must be considered that this enzyme has such a specificity type that it requires in

the back-bone of its substrate the groups underlined in the following formula of *l*-leucylglycylglycine



If this hypothesis is correct, the enzyme is an imidoendopeptidase or, in terms of an older terminology, an imidoproteinase. The existence of the specificity class of imidoproteinases has already been discussed (8). On the basis of experiments then available, chymotrypsin was assigned to this specificity class. However, it was found later that the enzymatic activity in question must be attributed to the aminoexopeptidase activity of chymotrypsin.

A closer investigation of the imidoendopeptidase which splits *l*-leucylglycylglycine is very desirable. Experiments performed in this laboratory by Dr. Joseph S. Fruton have shown that various animal tissues contain enzymes that hydrolyze *l*-leucylglycylglycine by a specific mechanism different from that of a leucine-aminoexopeptidase. The example discussed above of the imidoendopeptidase and the *l*-leucine-aminoexopeptidase represents a second case (12) of two proteolytic enzymes exhibiting different back-bone specificities but hydrolyzing the same substrate at identical linkages, *i.e.*, with the formation of the same split-products.

The hypothesis that a crude extract of intestinal mucosa contains, in addition to leucine-aminopeptidase, a second enzyme that splits *l*-leucylglycylglycine is in agreement with the data in Table I. The initial activities in the crude extract (Preparation A) indicate nearly twice the activity for *l*-leucylglycylglycine as for *l*-leucylglycine, whereas the purified enzyme (Preparation C) shows nearly equal activities towards the two substrates. Obviously, there has been removed during the purification an enzyme which acts upon *l*-leucylglycylglycine but very much less or not at all upon *l*-leucylglycine.

An enzyme of the specificity type characterized by Formula I would be expected also to hydrolyze other tripeptides containing a central glycine residue. It has already been mentioned that the partly purified enzyme preparation discussed in the present section acts indeed upon such tripeptides (Table VIII). However, our experimental material is by no means sufficient to prove that all these tripeptides are actually split by the same enzyme.

The hydrolysis of triglycine approximates a first order reaction more closely in the presence of Mn^{++} , because there is also contained in the enzyme solution an enzyme which slowly hydrolyzes glycylglycine and which is inhibited by Mn^{++} . This inhibition becomes noticeable in the 24 hour values (Table VIII).

The data for the hydrolysis of *l*-leucylglycylglycine closely resemble a zero order reaction, in the absence and in the presence of Mn^{++} . One possible explanation may be that we may have a low substrate concentration in relation to the enzyme concentration, a condition favoring zero order kinetics as recently emphasized by Van Slyke ((9) p 33). The hydrolysis of *l*-alanylglycylglycine also follows zero order kinetics.

TABLE VIII

Action of Glycine imidoendopeptidase Preparation on Various Substrates

Substrate concentration, 0.05 mm per cc, temperature 40°, pH 7.9 to 8.0, 0.02 M veronal buffer

Substrate	Enzyme concentration	Time	No Mn^{++} added			0.001 M Mn^{++} added		
			Hydrolysis	C	Average C	Hydrolysis	C	Average C
	mg protein N per cc	hrs	per cent			per cent		
<i>l</i> -Leucylglycylglycine	0.00317	0.5	13			13		
		1.0	26			26		
		1.5	44			37		
		2.0	52			51		
		3.0	76			71		
		24.0	124			106		
Triglycine	0.00635	0.5	29			29	0.79	
		1.0	51			51	0.81	
		2.0	81			74	0.77	
		3.0	92			88	0.81	0.80
		24.0	127			102		
Diglycyl- <i>l</i> -proline	0.00635	0.5	20	0.51		20	(0.51)	
		1.25	40	0.47		46	(0.56)	
		2.0	55	0.46		65	(0.60)	
		3.0	73	0.50	0.49	93		
		24.0	184			197		
Diglycyl- <i>l</i> -hydroxyproline	0.00635	0.5	16	0.40		17	0.42	
		1.0	31	0.42		32	0.44	
		2.0	50	0.40		51	0.41	
		3.0	65	0.40	0.40	65	0.40	0.42
		24.0	99			160		

The hydrolysis of diglycyl-*l*-proline yields good first order velocity constants for the first 3 hours only in the absence of Mn^{++} . The calculated first order constants rise, in the presence of the metal, much earlier. This rise is the result of the concurrent rapid splitting of the dipeptide formed during the hydrolysis of diglycylproline. This dipeptide must be glycyl-*l*-proline and cannot be diglycine, since the hydrolysis of the former by prolidase is activated by Mn^{++} , while the hydrolysis of glycylglycine is

inhibited by the metal. Thus, it follows that the hydrolysis of diglycyl-*l*-proline takes place at the peptide bond adjacent to the free amino group and results in the formation of glycine and glycyl-*l*-proline as the first products. Our plan to test this conclusion by the isolation of the primary split-products could not be verified under present conditions.

For the enzymatic hydrolysis of diglycyl-*l*-hydroxyproline, good and almost identical first order velocity constants were obtained in the absence and in the presence of Mn^{++} . It is probable that here, as in the case of diglycylproline, the hydrolysis occurs between the two glycine residues. With the low enzyme concentration employed, the glycylhydroxyproline is hydrolyzed rather slowly even in the presence of activating manganese.

The data on the two stereoisomeric forms of alanylglycylglycine, also reported in Table IX, indicate that the *l* form is split about 90 times as rapidly as the *d* form. It is, at present, not known whether the same enzyme is responsible for the hydrolysis of the two stereoisomers.

Table IX provides some comparative data concerning the action of a similar and weaker enzyme preparation upon several substrates. The enzyme preparation contains in all probability other enzymes besides *l*-prolidase and glycine-imidoendopeptidase. One is justified, however, to conclude from Table IX that the imidoendopeptidase, which acts upon triglycine, is much less active towards glycylglycine, glycylglycinamide, and the tetrapeptides diglycyl-*l*-leucylglycine and diglycyl-*l*-glutamylglycine. The free carboxyl in the tripeptide appears to have a rather beneficial influence upon the action of the imidoendopeptidase.

Action of Kidney Cathepsin on Proline and Hydroxyproline Peptides

A highly active preparation of swine kidney cathepsin prepared according to Fruton, Irving, and Bergmann (8) was found to hydrolyze carbobenzoxyglycyl-*l*-proline to the extent to 20 per cent of one peptide bond within a period of 45 hours at pH 4.7 and 40°, with citrate buffer and cysteine as the activator. When no cysteine was added, only about 5 per cent splitting occurred. No hydrolysis was observed of the substrates carbobenzoxyglycyl-*l*-hydroxyproline, carbobenzoxyglycyl-*l*-prolinamide, and carbobenzoxyglycyl-*l*-hydroxyprolinamide. To our knowledge, this is the first reported observation of the hydrolysis of a proline peptide by a catheptic enzyme.

Instability of Dipeptides Containing Proline or Hydroxyproline

Freshly prepared solutions of glycyl-*l*-proline and glycyl-*l*-hydroxyproline are hydrolyzed to 100 per cent by solutions of prolidase. When such solutions are kept at room temperature before the enzyme is added, their carboxyl titer steadily decreases and simultaneously their sensitivity to-

wards prohydase diminishes. For the study of these peptides it is therefore necessary to use freshly prepared solutions.

TABLE IX

Hydrolysis of Various Peptides by Extract of Intestinal Mucosa, Purified by Lead Acetate

Enzyme concentration, 0.214 mg of protein N per cc, no Mn^{++} added, temperature 40°, pH 7.8 to 8.0

Substrate	Time	Hydrolysis	C
	hrs	per cent	
<i>l</i> -Alanylglycine	18	77	0.0028
	26	88	0.0028
	44	103	
Glycyl- <i>l</i> -alanine	26	17	0.00024
	44	25	0.00022
	72	35	0.00020
	95	46	0.00022
Glycylglycine	19	14	0.00026
	68	40	0.00026
Glycylglycinamide	19	35	0.00077
	68	85	0.00094
Glycinamide	19	7	0.00014
	68	26	0.00015
Diglycyl- <i>l</i> -leucylglycine	18	35	0.00081
	26	44	0.00076
	44	62	0.00075
	95	114	
Diglycyl- <i>l</i> -glutamylglycine	18	40	0.00097
	26	52	0.00096
	44	86	
	72	120	
	95	144	
<i>l</i> -Alanylglycylglycine	0.5	35	0.029
	1.25	77	0.040
	1.5	83	0.040
<i>d</i> -Alanylglycylglycine	19	22	0.00044
	68	51	0.00035
Triglycine	0.5	31	0.025
	1.25	73	0.035
	1.5	77	0.033

It is likely that the apparent decrease in peptide concentration is due to a slow transformation of the dipeptides into diketopiperazines in the slightly alkaline solution (pH, 8.0). It is noteworthy that the diketopiperazine of glycylproline has been repeatedly isolated from acid protein hydrolysates. Moreover, we have isolated diketopiperazines when carbo-

benzoyglycyl-*l*-prolinamide and carbobenzoxyglycyl-*l*-hydroxyprolinamide were hydrogenated in acid solution. On removal of the carbobenzoxy group, ammonia is eliminated spontaneously and the diketopiperazine is formed under ring closure. Abderhalden and Nienburg (13) have found that carbobenzoxyprolylproline forms proline anhydride on hydrogenation. However, the hydrogenation of carbobenzoxyglycyl-*l*-proline and of carbobenzoxyglycyl-*l*-hydroxyproline yields the expected dipeptides.

TABLE X
Optical Rotation of Dipeptides Containing Glycine

The rotations of all peptides were determined in aqueous solution

Peptide (1)	Bibliographic reference No (2)	Specific rotation (3)	Molecular rotation (4)	Δ (a) (b) (5)
		<i>degrees</i>	<i>degrees</i>	<i>degrees</i>
Glycyl- <i>l</i> -leucine	16	-35.1	(a) -6,602	
<i>l</i> -Leucylglycine	17	+85.5	(b) +16,083	22,685
Glycyl- <i>l</i> -valine*	18	-19.17	(a) -3,430	
<i>l</i> -Valylglycine*	18	+93.5	(b) +16,278	19,718
Glycyl- <i>l</i> -alanine	19	-50.0	(a) -7,305	
<i>l</i> -Alanylglycine	20	+50.3	(b) +7,349	14,654
Glycyl- <i>l</i> -hydroxyproline	This paper	-128.4	(a) -24,152	
<i>l</i> -Hydroxyprolylglycine	" "	-22.4	(b) -4,213	19,939
Glycyl- <i>l</i> -proline	21	-113.8	(a) -19,535	
<i>l</i> -Prolylglycine†	22	-22.8	(b) -4,023	15,562

* These dipeptides, containing natural valine or natural alanine, were designated in the publication referred to in Column 2 as glycyl-*d*-valine, *d*-valylglycine, glycyl-*d*-alanine, and *d*-alanyl-glycine.

† This substance is described (22) as containing 1 molecule of water of crystallization.

Optical Rotation of Several Dipeptides Containing Glycine

The synthesis of glycyl-*l*-hydroxyproline by the carbobenzoxy method reported in the experimental section has yielded a preparation possessing an $[\alpha]_D^{20} = -128.4^\circ$ (in water), while Abderhalden and Koppel (14) had reported for this peptide an $[\alpha]_D^{20} = -50.79^\circ$ (in water). The difference may, at least in part, be attributed to the fact that Abderhalden and Koppel synthesized the dipeptide by means of the halogenacyl method. Fischer and Reif (15) have shown that the application of this method to halogenacyl derivatives of proline sometimes results in the formation of products which are isomeric with the expected peptides. Similar complications may be expected for the derivatives of hydroxyproline.

In order to obtain additional evidence, the difference between the rotation

of glycyl-*l*-hydroxyproline and *l*-hydroxyprolylglycine was calculated and the value thus obtained was compared with the values reported for other dipeptides containing an optically active aliphatic amino acid combined with glycine. It will be noted from the data reported in Table X that the peptides containing the glycine carbonyl combined with the nitrogen of an aliphatic monoaminomonocarboxylic acid have a specific rotation $90-120^\circ$ more negative than the respective peptides with a reciprocal sequence of the two amino acid residues. The difference of the molecular rotations is $14,600-22,700^\circ$.

The difference of the molecular rotations for the reciprocal peptides containing proline or hydroxyproline respectively is very similar when the specific rotation of -128.4° is accepted for glycyl-*l*-proline. The value of -50.8° , on the other hand, as reported by Abderhalden and Koppel, is clearly out of line with the other data recorded in Table X.

The authors wish to thank Mr. Stephen M. Nagy, who performed the elementary analyses, for his valuable assistance.

EXPERIMENTAL

Enzymatic Studies

The activity and purity of different enzyme samples were determined by the methods defined by Irving, Fruton, and Bergmann (6). Wherever possible, the activity was determined by measuring the first order velocity constant K on the substrate S for the enzyme concentration E , where E is expressed as mg of protein N per cc of test solution. The proteolytic coefficient $C = K/E$. Although in some cases the reaction did not adhere strictly to a first order reaction, the initial constants were used in order to obtain comparative data. 1 unit of enzymatic activity is the quantity of enzyme which splits the substrate with a K of 0.0020 under the conditions used. The procedure was that followed in previous reports from this laboratory. Hydrolyses were carried out in 2.5 cc volumetric flasks at a temperature of 40° . Hydrolysis was measured on 0.2 cc samples by the titration method of Grassmann and Heyde (23). The substrate was present in a concentration of 0.05 M per cc (with the sole exception of racemic substrates which were used in a concentration of 0.1 M per cc). The solutions were buffered by the addition of 0.5 cc of 0.1 M veronal buffer to the test solution.

Hydrolysis of l-Leucylglycylglycine by Lead Acetate-Treated Mucosa Extract

307 mg of *l*-leucylglycylglycine were dissolved in water and brought to pH 8 by addition of NaOH. Enzyme was added equivalent to 0.317 mg

of protein N and the volume brought to 25 cc. After 35 minutes at 40°, the titration samples showed that 100 per cent splitting of one peptide bond had occurred. The reaction was stopped by addition of 4 cc. of N HCl and 20 cc. of absolute alcohol. The solution was concentrated *in vacuo*, and the residue dissolved in hot 95 per cent alcohol. The slight precipitate was filtered off and the solution again concentrated to dryness. The residue was dissolved in 5 cc. of hot water and 393 mg. of sodium 2-bromotoluene 5-sulfonate were added.³ This is equivalent to 1.2 moles for the calculated amount of leucine, one-twenty-fifth of the total sample had been removed for estimation of the enzymatic splitting. After standing overnight at 0°, the solution was filtered and the mother liquor retained for the isolation of diglycine. Yield of dried leucine bromotoluene sulfonate, 420 mg., or 87 per cent of theory. The salt was recrystallized from hot water.

$C_{13}H_{16}NO_6SBr$	Calculated	C 40.8, H 5.3, N 3.7
382.3	Found	" 40.6, " 5.5, " 3.7

To the mother liquor and washings (8 cc.) there were added 529 mg. of 5-nitronaphthalene-1-sulfonic acid. This sulfonic acid forms a sparingly soluble salt with glycylglycine which crystallizes as rhombohedral plates. Crystallization was completed overnight at 0°. The characteristic square plates of the glycine salt were absent. Yield, 337 mg., or 73 per cent of theory. The diglycine nitronaphthalene sulfonate was recrystallized from hot water.

$C_{14}H_{16}N_2O_6S$	Calculated	C 43.6, H 3.9, N 10.9
385.3	Found	" 43.8, " 4.1, " 11.0

Glycyl-L-hydroxyproline

Carbobenzoylglycyl-L-hydroxyproline.—To a solution of 5 gm. of *L*-hydroxyproline in 25 cc. of 2 N NaOH there were added in portions with cooling and shaking an additional 20 cc. of 2 N NaOH and 10 gm. of carbobenzoylglycyl chloride. On acidification with concentrated HCl, the compound precipitated as an oil. The oil was extracted with ethyl acetate. This solution was washed with dilute HCl and with water and then dried over Na_2SO_4 . On concentration of this solution *in vacuo* to a thick syrup, sheaves of needles formed on standing at room temperature. The needles were filtered and washed with ether. Yield, 7.6 gm. On recrystallization from hot ethyl acetate, thin triangular plates were obtained. M_p 124–124.5°.

$C_{15}H_{15}O_6N$	Calculated	C 55.9, H 5.6, N 8.7
322.3	Found	" 56.0, " 5.7, " 8.5

³ The leucine salt of 2-bromotoluene 5-sulfonic acid (24) is considerably less soluble than the glycine, leucylglycine, and diglycine salts of this sulfonic acid.

Glycyl-l-hydroxyproline—4 gm of the carbobenzoxy compound were hydrogenated in the presence of palladium black in methyl alcohol containing 4 cc of water and 4 cc of glacial acetic acid. The only precipitate was dissolved by the addition of water and the catalyst filtered off. The solution was evaporated *in vacuo* with the addition of methanol. The syrup thus obtained crystallized on addition of absolute ethanol. Yield, 2.3 gm

$C_7H_9N_2O_4$	Calculated	C 44.7, H 6.4, N 14.9
188.1	Found	" 44.4, " 6.5, " 14.8
$[\alpha]_D^{25} = -128.4^\circ$ (1.8% in water)		

Diglycyl-l-hydroxyproline

l-Hydroxyproline Benzyl Ester Hydrochloride—25 gm of *l*-hydroxyproline were esterified twice with dry HCl in 350 cc of benzyl alcohol, followed by removal of HCl and water *in vacuo* at 85° . The insoluble residue was filtered off and the ester hydrochloride was crystallized from the mother liquor by adding dry ether. Yield, 18.5 gm of needles. They were dissolved in benzyl alcohol and crystallized by the addition of ether. M p, $147-150^\circ$

$C_{12}H_{16}NO_3Cl$	Calculated	C 55.9, H 6.3, N 5.4
257.7	Found	" 56.0, " 6.4, " 5.4

Carbobenzoxydiglycyl-l-hydroxyproline Benzyl Ester—The carbobenzoxyglycylglycinazide used for this synthesis was prepared from the corresponding hydrazide according to Rinke (25). It was partially dried on a porous plate and then dissolved in ethyl acetate. This solution was successively washed with ice water, aqueous bicarbonate, and then with water. The solution was dried over Na_2SO_4 and then added to a dry ethyl acetate solution of hydroxyproline benzyl ester prepared from 6.4 gm of the hydrochloride described in the preceding section. After standing at room temperature overnight, the solution was washed with water, dilute HCl, ~~water~~ dilute bicarbonate, and again with water, dried over Na_2SO_4 , and concentrated *in vacuo*. On addition of ether, the carbobenzoxy peptide ~~ester~~ crystallized in needles. It was recrystallized from ethyl acetate-~~ether~~ ether. Yield, 4.7 gm. M p, $123-127^\circ$

$C_{21}H_{27}N_3O_7$	Calculated	C 61.4, H 5.8, N 9.0
469.5	Found	" 61.4, " 5.9, " 8.9

Free Tripeptide—3 gm of the carbobenzoxy tripeptide ~~benzyl ester~~ was hydrogenated in the usual manner in methyl alcohol containing 4 cc of water and 2 cc of glacial acetic acid. After concentration ~~of the solution~~

solution *in vacuo*, the substance crystallized on addition of absolute alcohol
Yield, 1.5 gm It was recrystallized from water-alcohol

$C_9H_{15}N_3O_5 \cdot H_2O$	Calculated	C 41.1, H 6.5, N 16.0
263.2	Found	" 41.3, " 6.6, " 15.7
$[\alpha]_D^{25} = -97.7^\circ$ (2.8% in water)		

Diglycyl-L-proline

Carbobenzoxydiglycyl-L-proline Benzyl Ester—A dry ethyl acetate solution of L-proline benzyl ester⁴ from 6 gm of the hydrochloride was coupled in ethyl acetate solution with carbobenzoxyglycylglycinazide from 4.6 gm of the corresponding hydrazide and the solution purified in a manner similar to that described above for the preparation of the corresponding carbobenzoxydiglycyl-L-hydroxyproline benzyl ester. After the ethyl acetate solution was concentrated *in vacuo*, anhydrous ether and petroleum ether were added to the residue. The carbobenzoxy benzyl ester crystallized as needles. Yield, 5.2 gm. After recrystallization of the ester from ethyl acetate-petroleum ether, its melting point was 87° .

$C_{14}H_{17}N_3O_6$	Calculated	C 63.5, H 6.0, N 9.3
453.5	Found	" 63.5, " 6.3, " 9.3

Diglycyl-L-proline—4 gm of the carbobenzoxy tripeptide benzyl ester were hydrogenated in the usual way in methyl alcohol containing 3 cc of water and 3 cc of glacial acetic acid. After evaporation of the filtered solution and the addition of absolute ethanol, the tripeptide crystallized as rectangular plates. Yield, 1.9 gm.

$C_8H_{13}N_3O_4 + \frac{1}{2}H_2O$	Calculated	C 45.4, H 6.8, N 17.6, H_2O 3.8
238.2	Found	" 45.4, " 6.8, " 18.0, " 3.9
$[\alpha]_D^{25} = -101.5^\circ$ (2.2% in water)		

Carbobenzoxyglycyl-L-hydroxyprolinamide

L-Hydroxyproline Methyl Ester Hydrochloride—7 gm of L-hydroxyproline were twice esterified with dry HCl in methanol, the solvent being removed by concentration *in vacuo* each time. Yield, 8.4 gm of needles. The compound was twice recrystallized from methanol-ether. M_p , $162-164^\circ$, with evolution of gas.

$C_8H_{12}NO_3Cl$	Calculated	C 39.7, H 6.6, N 7.7
181.6	Found	" 39.5, " 6.9, " 7.8

⁴ The benzyl ester hydrochloride of L-proline was obtained by a procedure analogous to that described above for the benzyl ester hydrochloride of L-hydroxyproline. The proline ester, which crystallized readily from benzyl alcohol, could not be obtained analytically pure. The crude ester could, however, be used satisfactorily for syntheses.

Carbobenzoxyglycyl-l-hydroxyprolinamide—A dry solution in ethyl acetate of hydroxyproline methyl ester prepared from 5 gm of the hydrochloride was coupled with 7 gm of carbobenzoxyglycine chloride. The solution was washed with bicarbonate, water, and dilute HCl and dried over Na_2SO_4 . The solvent was removed *in vacuo* and the resulting syrup dissolved in absolute methyl alcohol previously saturated with dry NH_3 gas at 0° . This solution was allowed to stand for 2 days in a pressure flask at room temperature. The solvent was then removed *in vacuo*, and the amide crystallized from methanol-ether. *M p*, 208°

$\text{C}_{15}\text{H}_{19}\text{N}_3\text{O}_5$	Calculated	C 56.0, H 6.0, N 13.1
321.2	Found	" 55.9, " 5.9, " 12.9

Glycyl-l-hydroxyproline Diketopiperazine—3 gm of the above carbobenzoxy dipeptide amide were hydrogenated in the usual manner in methanol containing 3 cc of water and 3 cc of glacial acetic acid. On concentration of the filtrate *in vacuo* with the repeated addition of absolute methanol, 2.1 gm of large prisms were obtained. They were recrystallized from absolute methanol. This compound contained no amino nitrogen and gave the analytical values for the diketopiperazine

$\text{C}_7\text{H}_{10}\text{N}_2\text{O}_2$	Calculated	C 49.4, H 5.9, N 16.5
170.2	Found	" 49.4, " 6.0, " 16.5
$[\alpha]_D^{25} = -190.4^\circ$ (1.9% in water)		

Carbobenzoxyglycyl-l-prolinamide—A dry solution in ethyl acetate of *l*-proline benzyl ester from 6 gm of the hydrochloride was coupled with 7 gm of carbobenzoxyglycyl chloride. The solution was washed with bicarbonate, water, dilute HCl, and water, and then dried over Na_2SO_4 . The solvent was then removed *in vacuo*, and the syrup dissolved in absolute methanol which had been previously saturated with dry NH_3 gas at 0° . After standing for 3 days at room temperature in a stoppered flask, the solution was repeatedly concentrated *in vacuo* with methanol until it was free of NH_3 . Crystallization occurred on addition of ether and petroleum ether. Yield, 7.0 gm of needles. They were recrystallized from methanol by the addition of ether and petroleum ether. *M p*, 150 – 151°

$\text{C}_{15}\text{H}_{19}\text{N}_3\text{O}_4$	Calculated	C 59.0, H 6.2, N 13.8
305.1	Found	" 59.1, " 6.3, " 13.6

Glycyl-l-proline Diketopiperazine—3 gm of the above amide were hydrogenated in the usual way in methanol containing 3 cc of water and 3 cc of glacial acetic acid. The filtrate was concentrated *in vacuo* with the repeated addition of methanol. On addition of absolute alcohol the diketo-

piperazine crystallized as regular octahedra The substance contained no amino nitrogen M p, 213°

$C_7H_{10}N_2O_2$	Calculated	C 54.5, H 6.5, N 18.2
154.2	Found	" 54.4, " 6.7, " 18.2
$[\alpha]_D^{20} = -197.3^\circ$ (8.1% in water)		

Fischer and Reif (15) report a melting point of 213° and $[\alpha]_D^{20} = -217.4^\circ$ for this compound This compound has also been isolated from several different protein hydrolysates from gelatin by Levene and Beatty (26) who give a melting point of 182–183°, from ghadin by Abderhalden (27) who reports a melting point of 209° and $[\alpha]_D^{20} = -206.5^\circ$, and from edestin by Abderhalden and Komm (28) who give a melting point of 180–183° and $[\alpha]_D^{20} = -202^\circ$

l-Hydroxyprolylglycine

Carbobenzoxy-l-hydroxyproline Hydrazide—To 10 gm of hydroxyproline methyl ester hydrochloride in 50 cc of water with 100 cc of chloroform there were added in portions with cooling and shaking 3 gm of MgO and 15 gm of carbobenzoxy chloride 20 minutes after the last addition, 2 cc of pyridine were added 5 N HCl was then added until the aqueous layer reacted acid The chloroform layer was washed with water, bicarbonate, and dilute HCl, and dried over Na_2SO_4 The solution was then concentrated *in vacuo* with the repeated addition of alcohol The syrup thus obtained was dissolved in 50 cc of absolute alcohol, and filtered 3.8 gm of hydrazine hydrate were added and the solution allowed to stand at room temperature overnight The slight precipitate was filtered and the solution concentrated *in vacuo* repeatedly with ether Yield, 5.6 gm of needles They were recrystallized from ethyl acetate-ether M p, 149–149.5°

$C_{13}H_{17}N_3O_4$	Calculated	C 55.9, H 6.1, N 15.0
279.3	Found	" 55.9, " 6.0, " 15.0

Carbobenzoxy-l-hydroxyprolylglycine Benzyl Ester—5 gm of carbobenzoxy *l*-hydroxyproline hydrazide were converted to the azide by dissolving in 50 cc of water with 1 cc of concentrated HCl and 3 cc of glacial acetic acid A solution of 1.5 gm of sodium nitrite in 5 cc of water was added with cooling and shaking The oily precipitate was extracted into ethyl acetate and washed with cold water, bicarbonate, and again with water After drying, the solution of the azide was added to a dry ethyl acetate solution of glycine benzyl ester prepared from 5.4 gm of the hydrochloride, and the mixture allowed to stand overnight at room temperature The solution was concentrated *in vacuo* to yield 4.7 gm of needles The substance was twice recrystallized from ethyl acetate M p, 153°

$C_{22}H_{24}N_2O_6$	Calculated	C 64.1, H 5.9, N 6.8
412.4	Found	" 64.3, " 6.1, " 6.9

l-Hydroxyprolylglycine—3 gm of the above carbobenzoxy dipeptide benzyl ester were hydrogenated in the usual way in methanol containing 2 cc of glacial acetic acid and 2 cc of water. The peptide which crystallized during the hydrogenation was dissolved by the addition of a little water, and the solution was filtered and then repeatedly concentrated *in vacuo* with the addition of methanol. Yield, 1.3 gm of needles. The dipeptide was recrystallized from water-methanol.

$C_7H_{11}N_2O_4$	Calculated	C 44.7, H 6.4, N 14.9
188.1	Found	" 44.8, " 6.4, " 14.8
$[\alpha]_D^{25} = -22.42^\circ$ (7.7% in water)		

l-Prolinamide Hydrochloride—9 gm of *l*-proline benzyl ester hydrochloride were converted to the free ester in ethyl acetate in the usual manner. After drying, the solvent was evaporated off with the three times repeated addition of methanol. The syrup was dissolved in 75 cc of methanol, which was previously saturated with dry NH_3 gas at 0° . After standing at room temperature for 3 days in a stoppered flask, the solution was repeatedly concentrated with methanol. Yield, 3 gm of needles. These were dissolved in methanol and an excess of dilute HCl was added. This solution was repeatedly concentrated *in vacuo* with methanol. The needles of the amide hydrochloride were recrystallized from methanol-ether. M p, $173-175^\circ$.

$C_8H_{11}N_2OCl$	Calculated	C 39.9, H 7.4, N 18.6
150.6	Found	" 40.0, " 7.4, " 18.5

l-Hydroxyprolinamide—*l*-Hydroxyproline methyl ester was prepared in dry chloroform solution from 4.6 gm of the hydrochloride. The solution was concentrated *in vacuo*. Methanol was twice added and the solution again evaporated. The syrup was dissolved in 75 cc of methanol, and the solution saturated with dry NH_3 and kept in a stoppered flask for 2 days at room temperature. The solution was repeatedly evaporated *in vacuo*, with the addition of methanol. Yield, 2.2 gm of needles. They were recrystallized from ethyl acetate. M p, 139° .

$C_8H_{10}N_2O$	Calculated	C 46.1, H 7.8, N 21.5
130.2	Found	" 46.1, " 8.0, " 21.2

Carbobenzoxy-l-prolyl-l-proline—This was prepared as described by Abderhalden and Nienburg (13).

l-Alanylglycylglycine—This was prepared as described by Bergmann and Fruton (3). The carbobenzoxy-*l*-alanylglycine hydrazide used in the preparation of the tripeptide had, after recrystallization from hot water, a melting point of 157° . Bergmann and Fruton give for this compound a melting point of $145-147^\circ$.

Carbobenzoxylglycylglycine Hydrazide—This was prepared as described by Rinke (25)

Carbobenzoxylglycyl-l-proline and *Glycyl-l-proline*—These were prepared as described by Bergmann, Zervas, Schleich, and Lemert (21)

SUMMARY

1 The preparation of a *l*-leucine-aminoexopeptidase from aqueous extracts of swine intestinal mucosa is described. The purified enzyme protein is stable but becomes rather unstable when activated by Mn^{++} , thus frustrating attempts at establishing reliable proteolytic quotients.

2 Two procedures are described for the preparation of prolidase (substrates, glycyl-*l*-proline and glycyl-*l*-hydroxyproline) from intestinal mucosa. Prolidase is activated by Mn^{++} , but not by Mg^{++} , Co^{++} , Cu^{++} , or Zn^{++} . With the manganese-activated enzyme, satisfactory first order velocity constants were obtained.

3 Swine intestinal mucosa is found to contain one or several endopeptidases hydrolyzing carbobenzoxylglycyl-*l*-prolinamide and carbobenzoxylglycyl-*l*-hydroxyprolinamide.

4 Swine intestinal mucosa contains, in addition to *l*-leucine aminoexopeptidase, a second enzyme that hydrolyzes *l*-leucylglycylglycine to yield leucine and glycylglycine. In contrast to the leucinepeptidase, the new enzyme is not activated by Mn^{++} and acts not at all or only very slowly upon *l*-leucylglycine. It is tentatively classified as a glycine-imidoendopeptidase.

5 Swine kidney contains an enzyme which, in the presence of added cysteine, hydrolyzes carbobenzoxylglycyl-*l*-proline.

6 Evidence is offered for the presence in swine intestinal mucosa of enzymes hydrolyzing glycylglycine and glycyl-*l*-leucine, glycyl *l*-alanine, *l*-alanylglycine, *l*-prolylglycine and *l*-hydroxyprolylglycine, and *d*-leucylglycylglycine.

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ELECTROPHORESIS, SEDIMENTATION, AND ADENOSINETRIPHOSPHATASE ACTIVITY OF MYOSIN

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In 1939 Engelhart and Ljubimova (1) showed that myosin extracts were able to convert adenosine triphosphate into adenosine diphosphate and inorganic phosphate. This work has led to an interesting concept of the manner in which chemical energy is transformed into the mechanical energy of muscle contraction (2, 3). A number of investigators (1, 4) have attempted to separate the adenosinetriphosphatase activity of myosin extracts from the myosin by precipitation methods, but to date this has not been possible. The present communication reports on the electrophoretic migration of myosin in the Tiselius apparatus and the determination of the adenosinetriphosphatase activity of various fractions removed from the Tiselius cell following electrophoresis. Values for sedimentation and diffusion constants for myosin are also given.

EXPERIMENTAL

Preparation—Myosin was prepared from leg muscle of the rabbit by the method of Greenstein and Edsall (5). Adenosine triphosphate was prepared as the monobarium salt according to Kerr (6), and was converted to the sodium salt by treatment with sodium sulfate. Adenosinetriphosphatase was measured after treatment with glutathione by a method previously described (7), the activity being given in micrograms of inorganic phosphorus liberated enzymatically at 37° and pH 8.6.

Electrophoresis—The myosin preparations were analyzed at pH 6.2 in phosphate buffer, and at pH 7.4, 7.8, and 8.6 in borate buffer (see Table I). All the buffer solutions contained 0.4 M KCl. Electrophoretic patterns (Fig. 1) taken at the end of 6 hours (180 volts per cm) indicated a homogeneous material, but after electrophoresis for 16 to 20 hours (116 volts per cm, pH 7.8), a small fraction of lower mobility began to appear (Fig. 2, A), and after 67 hours the pattern of Fig. 2, B was obtained.

In order to determine whether the enzymatic activity was associated with the main electrophoretic component, samples taken at the end of three experiments were measured. These experiments were carried out at pH 7.80 in borate buffer containing 0.4 M KCl with a 10 ml Tiselius cell. In the first experiment after electrolysis for 16 hours, five 1 ml samples were taken

as follows Sample 1, buffer solution immediately above the ascending boundary, Sample 2, protein solution immediately below the ascending boundary, Sample 3, middle fraction from the bottom of the U-tube, Sample 4,

TABLE I
Mobilities of Myosin

Myosin concentration in Experiments 1, 2, and 4, 0.91 mg of N per ml, in Experiment 3, 1.04 mg of N per ml, the phosphate buffer contained 0.02 M PO_4^{3-} , 0.05 M NaCl, 0.40 M KCl, the borate buffer contained 0.11 M H_2BO_3 , 0.053 M NaOH, 0.048 M HCl, 0.40 M KCl

Experiment No	pH	Buffer	Mobility $\times 10^5$ cm ² per sec per volt descending
1	6.20	Phosphate	2.60
2	7.35	Borate	2.79
3	7.80	"	2.94
4	8.65	"	3.10

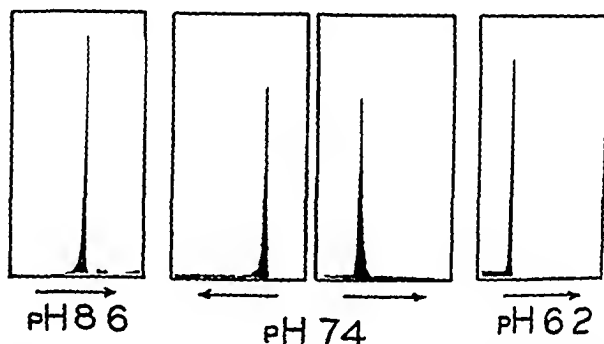


FIG 1 Electrophoresis patterns of myosin obtained after 6 hours at 1.80 volts per cm (arrows pointing right indicate descending patterns, left, ascending)

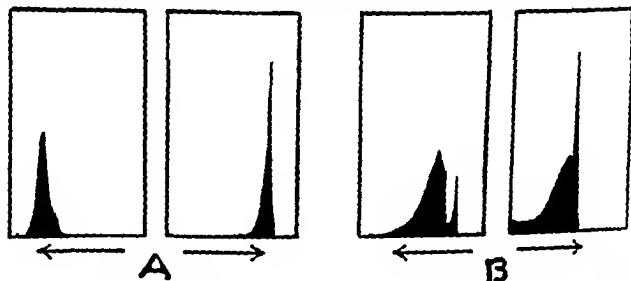


FIG 2 Electrophoresis patterns of myosin (4) after 16 hours and (B) after 67 hours, at 1.16 volts per cm (arrows pointing left indicate descending pattern, right, ascending)

protein solution immediately below the descending boundary, Sample 5, buffer solution immediately above the descending boundary. It was found that there was no adenosinetriphosphatase activity in the buffer solutions above either boundary and that the activity was uniformly distributed in the protein (see Table II).

In the other experiments in which electrophoresis with compensation was continued for 63 and for 67 hours respectively, the small, more slowly migrating component, constituting 15 per cent of the total protein as calculated from descending pattern areas, was effectively separated and a 0.5 ml sample of it was taken from the descending limb of the cell. Similarly a 0.5 ml sample of the fast component was taken from the ascending limb. Buffer solutions taken from above each of the boundaries were again found to be devoid of activity. Only 9 per cent of the total enzymatic activity

TABLE II

Adenosinetriphosphatase Activity of Myosin Samples Removed from Tiselius Cell Following Electrophoresis for 16 Hours

Myosin concentration of all samples, 0.59 mg of N per ml, borate buffer (see Table I), pH 7.80

Origin of sample	Adenosinetriphosphatase activity
Behind ascending boundary	23
Middle of U-tube	24
Ahead of descending boundary	24
Solution before electrophoresis	21

* Given in micrograms of inorganic phosphorus liberated enzymatically at 37° and pH 8.6

was associated with the slow component in one experiment (see Table III) and even less in the other. Determination of the specific activity per mg of the slow component was necessarily in some error because of the small amounts of protein obtainable and because of the probability of admixture of small quantities of the fast component. It is evident from Table III, therefore, that at least 90 per cent of the adenosinetriphosphatase activity is associated with the main electrophoretic component of myosin.

Ultracentrifugation—Two preparations of myosin, one containing 0.91 mg of N per ml and the other containing 0.52 mg of N per ml, were analyzed in the ultracentrifuge (8) at pH 7.8 in borate buffer (see Table I) containing 0.4 M KCl, and both were found to be monodisperse (see Fig. 3). A sedimentation constant of 9.5 Svedberg units after correction to pure water was calculated for the material at higher concentration and 12.0 for the material at lower concentration. Diffusion constants were measured

A METHOD FOR THE ASSAY OF RIBONUCLEINASE IN BIOLOGICAL MATERIAL*

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Ribonucleinase splits ribonucleic acid into smaller fragments which are diffusible through semipermeable membranes and which are no longer precipitable by dilute mineral acids. These fragments have been shown to be mononucleotides uridylic, cytidylic, guanylic, and adenylic acids (2). The enzyme has been studied in various stages of purity (3, 4) and has been isolated and crystallized (1). Few data are available upon the occurrence of ribonucleinase in tissue, and in view of the probably significant rôle that nucleoproteins hold in cellular economy (5) and the possible importance of this enzyme in their metabolism such information would be highly interesting. Methods used for quantitative estimation of the enzyme during the course of its purification involve the determination of "soluble phosphorus" or "diffusible phosphorus" (6, 1), but such techniques have not been used, nor do they seem suited, for the assay of whole tissue. Since the enzyme acts upon its substrate to liberate titratable acidic groups (1, 7), it was possible to develop a manometric method in which the acidic groups were made to evolve carbon dioxide from a bicarbonate buffer and the evolution of the gas followed by means of the conventional Warburg apparatus. This method and its application to the assay of various tissues are described herein.

EXPERIMENTAL

Development of Method—The details of the method were first worked out with a crystalline enzyme prepared according to Kunitz¹ (1). Purified yeast nucleic acid was used as a substrate. Purification was accomplished by precipitating commercial nucleic acid² with glacial acetic acid, washing four times with water, once with ethyl alcohol, once with ethyl ether, and

* The enzyme, which was provisionally named "ribonuclease" by Kunitz (1), has been designated throughout this paper as "ribonucleinase" according to the suggestion of Loring and Carpenter (2).

This investigation was aided by a grant from the Jonathan Bowman Fund for Cancer Research.

¹ The crystalline enzyme was prepared by Mr. Gerald C. Mueller of this laboratory. We are also indebted to Dr. Kunitz for a sample preparation.

² Pfanstiehl Chemical Company, Waukegan, Illinois.

then drying in a vacuum desiccator. This was an essential procedure, because with unpurified preparations the rate of carbon dioxide evolution falls off rapidly, possibly from inhibition of the reaction by decomposition products present in the crude nucleic acid.

The components of the test system were as follows: 10 ml of 0.1 M NaHCO_3 , 2.0 ml of yeast nucleic acid solution containing 100 mg per ml, and 0.1 ml of crystalline enzyme solution containing 0.2 mg per ml.

The above solutions were pipetted into the Warburg reaction vessel, the enzyme solution being placed in the side arm. The apparatus was thoroughly gassed with a 5 per cent CO_2 -95 per cent O_2 mixture, equilibrated for 10 minutes at 37° , zero readings taken, and the enzyme solution tipped from the side arm. The first 5 minute reading was discarded and the next two or three readings were used to determine the rate. Although the amount of

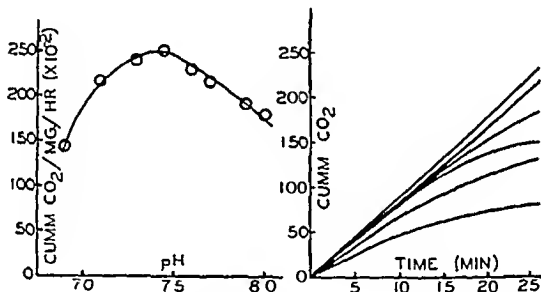


FIG 1

FIG 2

Fig 1 Effect of pH on ribonuclease activity

Fig 2 Effect of variation of substrate level on ribonuclease activity. Beginning with the highest, the curves represent 200, 150, 75, 50, and 25 mg of substrate per flask respectively.

CO_2 liberated is dependent on the purity of the enzyme preparation, a typical system gave about 30 c mm of CO_2 per 5 minute interval at a linear rate for a period of 20 to 30 minutes.

The pH of the medium was controlled by varying the concentration of the NaHCO_3 used. When subjected to a constant $p\text{CO}_2$, the pH of a medium containing NaHCO_3 , other factors being constant, is a function of the concentration of the bicarbonate following the Henderson-Hasselbalch relationship. The bicarbonate concentration was varied from 0.0009 to 0.066 final molarity in appropriate steps. The pH was checked with a glass electrode at the end of a 20 minute run to detect variations from the calculated values. The results were recorded in the form of a curve (Fig 1), from which it will be seen that the optimum pH is approximately 7.5, in

agreement with Kunitz (1) who used the "soluble phosphorus" method to follow activity

In this method of assay it is necessary to be certain that sufficient substrate is present to saturate the enzyme throughout the period of measurement (8). Substrate levels were therefore varied from 25 to 200 mg per flask in appropriate increments by use of the system described above. Results presented in Fig 2 demonstrate that 200 mg of nucleic acid were more than enough to saturate 0.01 mg of crystalline enzyme for at least 20 minutes.

In order to determine whether the rate of carbon dioxide production was proportional to the amount of enzyme present, the enzyme levels were varied from 0.01 to 0.1 mg per flask in a system containing 300 mg of nucleic acid. The results are shown in Fig 3, where rate is plotted against enzyme concentration. A straight line was obtained from 0.01 to 0.05 mg

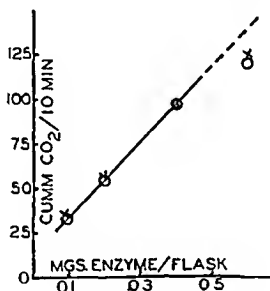


FIG 3 Relation between rate of carbon dioxide evolution and ribonuclease concentration

of enzyme per flask, which indicates that proportionality exists between enzyme concentration and the rate of gas production within this range.

Preliminary investigations on the effects of inorganic ions indicated that aluminum, potassium, manganese, barium, chloride, and sulfate at final molarities of 0.001, and magnesium and calcium at levels of 0.004 final molarity had no effect upon the system. Copper and zinc, however, gave inhibitions up to 50 per cent at final molarities of 0.001.

In the experiments described thus far no attempt was made to correct for the retention of carbon dioxide by the medium, since the errors involved were either small or compensatory and would make little difference in the over-all picture obtained. However, if the method is to be used as an enzyme assay, particularly in tissue in which there are a number of buffers, a correction must be applied in order to obtain accurate estimates of the amount of enzyme present. The retention correction for a given system is

most easily obtained by direct methods (8) Warburg flasks, so designed as to allow the addition of two substances to the system at different times, are required. Flasks with two side arms were found to be convenient. In one side arm was placed a measured amount of standard citric acid. The flask was then placed in an oven at 75° until the acid was completely dried. This was done in order to avoid changes in volume when the standard acid was added to the system. In the other side arm was placed the requisite amount of substrate solution, while the main part of the flask contained the tissue to be assayed, the NaHCO_3 buffer, and water to make a total volume of 3.0 ml. The flask was gassed and equilibrated in the usual manner and the substrate tipped in. The rate was followed for two 5 minute periods, at the end of the second period the dried acid was washed into the main part of the flask and three more readings taken. The first two and the last two readings gave the steady rate of the system. The difference between this rate and the third reading gave the amount of carbon dioxide evolved by the standard acid. The variation between this value and the value obtained by dumping standard acid into NaHCO_3 alone allowed a calculation of the retention correction to be made. The amount of acid introduced into the system was not large enough to change the pH and thus the activity of the enzyme was not affected. The correction obtained is a function not only of the buffering capacity of the medium, but also of the volume of the flask and the amount of the gas evolved (9).

In the conventional method (9) each flask is calibrated individually. A method has been devised whereby any number of flasks may be calibrated for retention from the data obtained by the use of just one flask, provided the volume of each is known.

If the flask constants (k) are calculated for a series of flasks in the usual manner, $\alpha = 0.550$ (9), and plotted against the gas volumes (V_g), a straight line, hereafter referred to as the base-line, is obtained (Fig. 4, Curve I). Suppose that a medium which retains carbon dioxide is introduced into these flasks. Each flask will now have a constant which is equal to k plus an amount r which will vary with each flask. If these new k values are plotted against V_g as before, a straight line will again be obtained, but will be found to lie above the base-line and to have a different slope (Fig. 4, Curve II). The characteristics of this line are reflections of the fact that the amount of retention is a function of the buffering capacity of the medium, the volume of the flask, and the amount of gas evolved.

By introducing a given medium into three flasks of different volumes and measuring the amount of CO_2 given off when a known amount of standard acid was added, a retention line was determined directly. This was done with several media of different retentions and the data plotted as shown in Fig. 4, Curves II, III, and IV. It was found that the slope (m) of these

lines was proportional to the value of k at a given volume, i.e., when V_g is constant,

$$(1) \quad k_I m_{II} = k_{II} m_I$$

$$(2) \quad k_I m_{III} = k_{III} m_I$$

$$(3) \quad k_I m_{IV} = k_{IV} m_I$$

With this fact established (Table I) it became apparent that the retention line for a given medium could be determined from the data of one flask if the

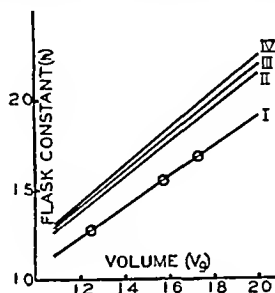


FIG 4 Carbon dioxide retention curves Curve I is the base line (see the text) Curves II, III, and IV are retention lines for media of varying retentions

TABLE I

*Relation between Slopes and Ordinates of Series of Carbon Dioxide Retention Curves**

Curve No	m	k	$k_I m_x$	$k_x m_I$
I	0.875	1.400		
II	0.975	1.575	1.365	1.375
III	1.000	1.610	1.400	1.410
IV	1.025	1.645	1.435	1.440

* See Fig 4

base-line and the volume of the test flask were known. The method of arriving at this conclusion is demonstrated as follows:

From Dixon (9) $k = x/h$, where k = the constant of the test flask of volume V_g , x = the theoretical c mm of carbon dioxide evolved by n ml of standard acid, and h = the manometer reading produced by n ml of standard acid.

Solving Equation 1 for m , we find $m = k m_{\text{base}} / k_{\text{base}}$, where m is the slope of the retention line, m_{base} the slope of the base-line, and k and k_{base} the constants of the flask of volume V_g at retention and at base-line levels.

And solving a simple analytical equation for a straight line, we find $k' = m(V'_g - V_g) + k$, where k' is the constant of the flask of volume V'_g at retention line level.

With V_p known and V'_p arbitrarily assigned, k and k' can be calculated from the above equations and the retention line thus defined by the points (k, V_p) and (k', V'_p) constructed. The constant for any apparatus volume may now be read directly from the retention curve for the medium involved.

Assay of Tissues—The method described above has been utilized in estimating the amount of ribonuclease occurring in various tissues of the rat. A water homogenate of the tissue to be assayed was prepared by use of the homogenizer described by Potter and Elvehjem (10). The strength of the homogenate was adjusted according to its content of ribonuclease to give rates corresponding to the linear range of Fig. 3. The equilibration period was lengthened in order to eliminate effects of endogenous oxygen uptake, and as a further check on this point a control flask without substrate was used. The same end can be better obtained by using a gas mixture containing an inert gas instead of the 5 per cent CO_2 -95 per cent O_2 mixture, but unfortunately the former was not available at the time most of the experiments were run. It was found, however, that lengthening the equilibration period practically eliminated endogenous effects. Since the ribonuclease is remarkably stable (6, 1), this additional treatment did not change the enzyme activity, experiments on spleen and kidney with 5 per cent CO_2 -95 per cent N_2 yielding the same results as those in which 5 per cent CO_2 -95 per cent O_2 was employed. All assays on liver were carried out with 5 per cent CO_2 -95 per cent N_2 .

The complete assay set-up, therefore, consisted of three flasks charged as follows. Flask 1, nucleic acid (side arm), NaHCO_3 buffer, tissue, water, Flask 2, nucleic acid (side arm), NaHCO_3 buffer, tissue, water, standard acid (second side arm), Flask 3, NaHCO_3 buffer, tissue, water.

Flask 1 was the assay flask proper. Flask 2 served to determine the retention of carbon dioxide, and the rate before and after the addition of the standard acid provides duplication of Flask 1. Flask 3 is a control on endogenous effects.

Variation over an appropriate range of the amount of tissue used per flask demonstrated that the CO_2 output was directly proportional in every case to the mg. of tissue used.

Bolomey and Allen (11) have reported that the action of a non specific phosphatase preparation upon nucleic acid is increased 50 to 150 per cent following preliminary treatment of the acid with ribonuclease. Fluoride and cyanide have been reported to inhibit acid and alkaline phosphatases respectively (12, 13) and their inhibitory action on the phosphatase activity of various homogenates at pH 7.4 was confirmed in this laboratory. To determine whether or not phosphatase activity was affecting the results of the ribonuclease assay $\text{M}/150$ NaF and NaCN were added to a duplicate of Flask 1 in the assay set-up. Typical results are given in Table II. These data show that $\text{M}/150$ NaF and NaCN do not inhibit crystalline

ribonucleinase activity and that there is no significant difference between the activity of the inhibitor treated and natural homogenates, indicating that non-specific phosphatases are not an important source of error in the assay

TABLE II
Effect of Phosphatase Inhibitors upon Ribonucleinase Assay

Tissue	Rate (duplicate determinations)
	<i>microliters CO₂ per 5 min</i>
Crystalline ribonucleinase	14 5, 15 0
" " + M/150 NaF and NaCN	15 0, 15 0
Kidney	12 0, 12 5
" + M/150 NaF and NaCN	12 0, 12 0
Spleen	10 0, 10 0
" + M/150 NaF and NaCN	11 0
Pancreas	32 0, 30 0
" + M/150 NaF and NaCN	29 0
Liver	6 0 6 0
" + M/150 NaF and NaCN	6 5 6 5

TABLE III
Ribonucleinase Content of Rat Tissues and Various Tumors

The values are expressed as c mm per mg (wet weight) per hour

Tissue	No of samples	Range	Average
Pancreas	8	13 00-20 04	16 70
Spleen	12	1 80-2 49	2 06
Kidney	10	1 44-1 85	1 63
Submaxillary	5	0 90-1 02	0 98
Lung	5	0 65-1 20	0 98
Liver	5	0 33-0 41	0 37
Cardiac muscle	2	0 36-0 36	0 36
Skeletal "	2	0 30-0 36	0 33
Walker carcinosarcoma No 256*	5	0 96-1 20	1 06
Flexner-Jobling carcinoma*	5	0 48-0 60	0 55
Yale No 1 adenocarcinoma†	5	0 72-0 84	0 75
Fibrosarcoma†	5	0 42-0 66	0 51

* Rat tumor

† Mouse tumor

DISCUSSION

Table III summarizes the results obtained upon eight normal rat tissues and four tumors. It will be seen that the values obtained for pancreas were very much higher than any of the other tissues assayed. The variation between different animals was also quite marked in the case of this tissue. The latter circumstance was probably due to the fact that it was

extremely difficult to sample the organ satisfactorily because of its diffuse nature. The head of the pancreas of the rat was more active than the tail, and the values given are for the head as nearly as it could be obtained by macro dissection.

Preliminary determinations indicate that lymph gland, brain, and whole blood also contain appreciable amounts of ribonucleinase.

There are a few points of special interest with regard to the data presented in Table III. As may be seen, the ribonucleinase content is much higher in the glandular than in non-glandular tissues. This may be associated with the differences in the respective functions of these tissues. In addition, these results show a rough correlation with the ribonucleic acid content of tissues, pancreas is rich in this acid, while muscles are poorly supplied (14). There seem to be no striking features about the amount of ribonucleinase in tumor tissue as compared to normal tissue. The same type of result has been reported by Greenstein and Thompson (15) for ribonucleodepolymerase and thymonucleodepolymerase, other enzymes which are known to act upon nucleic acids. No difference in the activity of these two enzymes was found in normal and neoplastic tissues. However, the Walker carcinoma No. 256, which is probably the fastest growing and the most malignant of the four tumors assayed, does have the highest ribonucleinase content. Further speculation must wait upon a more specific knowledge of the function of this enzyme in the processes of cellular physiology.

SUMMARY

A manometric method for the determination of ribonucleinase in animal tissue is described and a simple procedure for the correction of carbon dioxide retention is also given.

The method has been applied to the assay of a number of normal and neoplastic tissues of the rat. Pancreas was found to be relatively high, while spleen, kidney, submaxillary, lung, liver, and muscle followed in that order. No important difference was found between the ribonucleinase activity of normal and neoplastic tissue.

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quite readily at pH 4.9 and room temperature, the buffer and the disodium phenyl phosphate solutions were prepared separately and mixed just before use.

Results

The results in both the short and long term series of experiments are very similar. The phosphatases of the liver and intestine are not affected either by castration or testosterone propionate treatment. Therefore, the values for these tissues have been omitted to save space. The enzymes of the kidneys, however, showed significant changes as a result of both castration and testosterone propionate therapy.

The kidneys decreased in size as a result of castration and hypertrophied as a result of testosterone propionate treatment (Table I). These two phenomena now have been observed by several groups of investigators (13-16).

Kidney "Alkaline" Phosphatase—The total "alkaline" phosphatase content of the kidneys of the castrated mice decreased in both experiments (Table I) but the amount per gm. of tissue showed no change in the 35 day experiment and increased somewhat but probably not significantly in the 115 day experiment.

The kidneys of both the castrated and normal mice treated with testosterone propionate show very similar and large decreases in "alkaline" phosphatase when calculated either as per gm. of tissue or total content.

Similar changes were obtained in the kidneys of normal female mice of the same age when treated with testosterone propionate pellets.

Kidney "Acid" Phosphatase—In contrast to the "alkaline" phosphatase, the "acid" phosphatase increases in the kidneys of the testosterone propionate-treated mice (Table I). Furthermore, the increase is greater in the animals treated over the longer period of time and the values per gm. of tissue now also are significantly increased.

It is noteworthy that the enzymes of the kidneys of both the normal and castrated mice responded in a similar manner to testosterone propionate treatment.

Withdrawal of Testosterone Propionate—At autopsy of the mice in the long term experiments, no pellets were recovered from the implantation sites in three of the treated normal animals. The hormone apparently had been completely absorbed only a few days previously, because the kidneys and seminal vesicles and prostates showed the same increases in size as those of the mice with intact pellets. Furthermore, the "alkaline" phosphatase showed the same decrease in activity but the "acid" phosphatase no longer was increased, it had decreased to 3.4, 3.6, and 3.8 units per gm., or less than half that of the intact animals and somewhat less than that of the castrated and normal controls. Since no such low values were

TABLE I
Effect of Castration and Testosterone Propionate (T P) on "Alkaline"* and "Acid"† Phosphatases of Mouse Kidney

Duration of treatment	Treatment	No. of mice	Testosterone propionate absorbed	Semi-nal vesicles and prostates	Kidneys	Alkaline phosphatase‡				Acid phosphatase‡			
						Total		Per gm		Total		Per gm	
						units	per cent changes§	units	per cent changes§	units	per cent changes§	units	per cent changes§
days			mg	mg	mg								
35	Normal	6		124	267	60 (53-71)	-25	202 (174-250)	-1	1 96 (1 62-2 50)	-7	6 72 (5 52-9 63)	+17
	Castrate	7		8	225	45 (43-48)	-62	200 (160-232)	-75	1 82 (1 33-2 78)	+61	7 89 (5 82-10 18)	+8
	Normal + T P	6	6 8	238	445	23 (22-26)	-65	50 (47-57)	-78	2 93 (2 62-3 38)	+50	7 54 (6 28-9 47)	+12
	Castrate + T P	6	8 0	290	399	21 (19-23)	-25	45 (43-49)	-70	3 82 (3 41-4 10)	+80	6 10 (4 32-7 28)	+32
115	Normal	6		206	395	104 (92-125)	-45	263 (229-312)	-64	1 82 (1 71-2 00)	-34	4 63 (4 12-5 30)	+2
	Castrate	9		8	255	78 (67-91)	-55	311 (262-364)	-45	1 21 (1 01-1 41)	+110	4 74 (3 72-5 49)	+54
	Normal + T P	6	13 7	288	561	47 (35-59)	-45	79 (65-102)	-45	3 28 (2 53-4 00)	+80	7 13 (6 80-7 31)	+32
	Castrate + T P	6	12 8	318	540	57 (50-72)	-45	105 (91-115)	-64	3 28 (2 53-4 00)	+80	6 10 (4 32-7 28)	+32

* 1 unit is that amount of phosphatase activity which at pH 9.8 and 37° will liberate 1 mg of phenol from the disodium phenyl phosphate substrate in 1 hour

† 1 unit is that amount of phosphatase activity which at pH 4.9 and 37° will liberate 1 mg of phenol from the disodium phenyl phosphate substrate in 1 hour

‡ The figures in parentheses indicate the range of values

§ Per cent of averages The changes are from normal control values

|| Average of three mice See the text, "Withdrawal of testosterone propionate"

obtained in either the normal or castrated mice with pellets intact at autopsy, it may be assumed, with due regard for the small number of animals, that the "acid" phosphatase values of the kidneys of the mice that lost their pellets was also increased but decreased after loss of stimulation by the androgen. Therefore, since the kidney weights of these mice did not change, it would seem that the effect of testosterone propionate on "acid" phosphatase is probably a direct stimulation of the cells to produce more of this enzyme, while the effect of the androgen on "alkaline" phosphatase is associated with the increase in size of the kidney.

Phosphatases of Tissues of Normal Mouse—In order to show the distribution of the enzymes in the various tissues, the values obtained for the

TABLE II
Comparison between "Alkaline"* and "Acid"† Phosphatases of Tissues of Normal Mouse

Tissue	Age 75 days			Age 155 days		
	Alkaline	Acid	Alkaline Acid	Alkaline	Acid	Alkaline Acid
	units per gm	units per gm		units per gm	units per gm	
Kidney	202.0	6.7	30.1	263.0	4.6	57.1
Intestine	66.0	4.6	14.4	117.0	5.6	20.9
Liver	8.8	6.4	1.4†	11.4	5.6	2.0‡

* 1 unit is that amount of phosphatase activity which at pH 9.8 and 37° will liberate 1 mg. of phenol from the disodium phenyl phosphate substrate in 1 hour.

† 1 unit is that amount of phosphatase activity which at pH 4.9 and 37° will liberate 1 mg. of phenol from the disodium phenyl phosphate substrate in 1 hour.

‡ The "acid" to "alkaline" phosphatase ratio of the liver varies with strains and species. A mixed albino strain of mice had an "acid" to "alkaline" phosphatase ratio of 2.1 in the liver, and both male and female rats of our colony from Wistar stock had an "acid" to "alkaline" phosphatase ratio of 15.1 in the liver.

organs of the normal animals are presented in Table II. The amounts of "acid" phosphatase in all of the tissues are small and of about the same order of magnitude. The "alkaline" phosphatase, on the other hand, occurs in large amounts in the kidneys and intestine, while the liver contains smaller amounts.³

Age and Amount of Phosphatases—All the organs of the older animals contain somewhat greater amounts of "alkaline" phosphatase than those of the younger mice (Table II).

³ The "acid" to "alkaline" phosphatase ratio of the liver varies with strains and species. A mixed albino strain of mice had an "acid" to "alkaline" phosphatase ratio of 2.1 in the liver, and both male and female rats of our colony from Wistar stock had an "acid" to "alkaline" phosphatase ratio of 15.1 in the liver.

The "acid" phosphatase of the kidney and liver is somewhat less and that of the intestine greater in the older animals

DISCUSSION

Although testosterone propionate produces changes in the phosphatases of the kidneys, it does not affect the enzymes of the liver or intestine. It might be assumed, therefore, that the kidney, in order to retain phosphorus to satisfy metabolic demands imposed by testosterone propionate (1-9), decreases its "alkaline" but increases its "acid" phosphatase content. The decrease in "alkaline" phosphatase probably is of greater importance than the increase in "acid" phosphatase because of the much greater amount of the former.

It is impossible to state at present in precisely what structures of the kidney the phosphatase changes occur. Experiments now being completed by the Gomori histochemical techniques (17) are hoped to elucidate this point and provide further information as to the purpose of the changes in enzyme activities.

The phosphatases of the mouse kidneys are not the only enzymes affected by testosterone propionate treatment. The arginase⁴ and *d*-amino acid oxidase (18) content of this organ are increased in even greater amounts than the changes noted in the phosphatases.

SUMMARY

There was a decrease in the "alkaline" (pH 9.8) phosphatase accompanied by an increase in the "acid" phosphatase (pH 4.9) in the kidneys of normal and castrated mice treated for 35 and 115 days with a subcutaneous pellet of testosterone propionate. Castration resulted in a decrease in both of the enzymes in about the same proportion as the diminution in kidney weight.

The enzymes of the liver and intestine were not significantly changed as a result of testosterone propionate treatment or castration.

The tissues of the older mice contained more "alkaline" phosphatase than those of the younger animals.

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CHEMICAL ANALYSIS OF THE INFLUENZA VIRUSES A (PR8 STRAIN) AND B (LEE STRAIN) AND THE SWINE INFLUENZA VIRUS*

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The isolation and purification of particulate components biologically characteristic of influenza virus A (PR8 strain) (1), influenza virus B (Lee strain) (2), and the swine influenza virus (3) have been reported. These components were obtained, by adsorption on and elution from chicken red blood cells combined with ultracentrifugation or by ultracentrifugation alone, from chorioallantoic fluid of chicken embryos infected with the respective types of the influenza virus. By these procedures concentrates of the particles were obtained in preparations of a high degree of homogeneity with respect to particle kind, with characteristic sedimentation constants and average particle sizes, as determined from sedimentation data and from electron micrographs. Preliminary chemical analyses, already reported briefly, showed the three types of influenza virus to be constituted of lipoprotein-carbohydrate complexes containing nucleic acid of the desoxyribose type. The present paper is concerned with a detailed description and comparison of the analyses made on influenza viruses A (PR8 strain) and B (Lee strain) and the swine influenza virus.

Material and Methods

In the previous work with the three types of virus, various modifications in methods and technique were introduced or discarded from time to time. For the present work a uniform technique was adopted for obtaining the concentrates of the three types of virus in order to have a better basis for comparing the results of the analyses. The strains of the influenza viruses employed for analysis were the same as those already described in the reports on the studies on purification (1-3).

For virus cultivation a 10^{-3} dilution in hormone broth of previous passage virus-infected chorioallantoic fluid was inoculated in 0.05 ml

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volumes into the chorioallantoic cavity of chick embryos of 11 days incubation at 37.5°. After 42 hours incubation at 37° following inoculation, the virus infected chorioallantoic fluid was drawn off, chilled in vessels immersed in cracked ice, and then spun in the angle centrifuge (in collodion tubes 3.5 inches long and 1.0 inch in diameter in a conical head, International centrifuge No. 2) at 2000 *g* for 10 minutes. Groups of 800 to 1000 chick embryos were used at a time to provide the pools of chorioallantoic fluid sufficient to yield in a single batch the quantity of the respective types of purified virus adequate for analysis (100 to 200 mg.)

Purification was effected in all instances by the combined procedures of adsorption on and elution from washed chicken red blood cells (4, 5) followed by ultracentrifugation. It has seemed that this method has given better final concentrates, as judged by analysis in the ultracentrifuge and examination in the electron microscope, than ultracentrifugation alone. The step of preliminary dialysis against Ringer-calcium chloride solution (1), frequently used in the earlier work to remove amorphous urates and inorganic salts, was omitted. This procedure was impractical with large volumes of chorioallantoic fluid, and its principal advantage, the apparent dispersing action and prevention of marked aggregation of the virus particles when they were sedimented at 27,000 *g*, was supplanted by the reduction of the centrifugal field to 20,000 *g* for final sedimentation. Under the influence of this field the virus sedimented readily, but the pellets were not as tenaciously packed as when sedimentation was carried out at 27,000 *g*. Pellets sedimented at 20,000 *g* redispersed quickly in Ringer-calcium chloride solution (1), which was used as the dispersion medium after ultracentrifugation.

The chilled chorioallantoic fluid, after angle centrifugation, was parceled in 200 ml. lots in 250 ml. centrifuge bottles, and the virus was adsorbed on washed, adult chicken red blood cells, slowly added with stirring. For the influenza virus A and the swine influenza virus, 3 ml. of packed cells per 100 ml. of fluid were used. With the influenza virus B, which does not agglutinate red blood cells as well or as massively as the influenza virus A or the swine virus, 4 ml. of packed cells per 100 ml. were used. After 2 minutes of adsorption and stirring, the bottles were immersed in cracked ice for 10 minutes, and then the massed cells were rapidly sedimented at 400 *g* in the horizontal centrifuge. The supernatant fluids were syphoned off and discarded. The cells from four bottles were washed into one with 200 ml. of chilled Ringer's solution and resedimented at 400 *g*. Elution was then carried out at 25° in a volume of Ringer's solution one-eighth or one-tenth that of the starting chorioallantoic fluid. 2 to 3 hours of elution were sufficient for recovery of the influenza viruses A and B, but the elution period required for the swine virus was 4 to 6 hours.

The eluates, after removal of the gross quantity of red blood cells, were spun in the angle centrifuge at 2000 *g* to free them of cell debris. The bright, opalescent eluates were then parceled in 15 ml collodion ultracentrifuge tubes and spun for 1 hour at 20,000 *g* in the quantity ultracentrifuge. The supernatant fluids were poured off, the inside walls of the tubes wiped with gauze, and the clear, gel-like pellets dissolved in Ringer-calcium chloride solution, adjusted to pH 7.3 (with 0.01 *N* NaOH). The concentration at which the pellets were taken up was 75 to 150 times with respect to the volume of the starting fluid, depending upon the yield. The concentrates were cleared of extraneous particles and aggregates by spinning at 5000 *g* in the ultracentrifuge for 5 minutes.

The 50 per cent point infectious units of concentrates of the respective types of the virus, when inoculated in 0.05 ml volumes in chick embryos of 11 days incubation, were influenza virus A, $10^{-12.4}$, influenza virus B, $10^{-11.6}$, and swine influenza virus $10^{-12.74}$ gm of virus per inoculum. The corresponding hemagglutinative activities were such that $10^{-6.5}$ of influenza virus A, $10^{-6.4}$ of influenza virus B, and $10^{-6.3}$ gm of the swine influenza virus gave the 2+ end-point.

The concentrates selected for analysis exhibited a single, slightly diffuse boundary in the analytical ultracentrifuge and a high degree of uniformity of particle kind when examined in the electron microscope.

Qualitative Examination of Concentrates—The final concentrates of the three types of virus containing 2.0 to 5.0 mg of virus per ml were bluish and opalescent. In the higher concentrations a marked yellow tint was observed with transmitted light. Solutions of the swine influenza virus have consistently appeared to be clearer and have exhibited a brighter opalescence than those of the two human types in comparable concentrations. Solutions have stood for long periods at 2–5° (3 to 4 months) without visible change or alteration in sedimentation characteristics and appearance in electron micrographs.

Concentrates heated gradually (2° per minute) in the water bath coagulated at 56–60°, the solution becoming at first opalescent and then very curdy. The coagulum was insoluble in water, 0.9 per cent sodium chloride solution, dilute HCl (0.1 *N*), and dilute NaOH (0.1 *N*). The coagulum remained insoluble also in 2 *N* NaOH, even with prolonged heating at 100°.

The usual protein tests, biuret, xanthoproteic, ninhydrin, and Millon's, were positive. The Molisch test in all cases was initially negative, but with hydrolysis, on standing, bound carbohydrate was freed, rendering the test positive. Positive tests for pentose with orcinol and sulfuric acid and with Bial's reagent were obtained for the three types of virus. The Bial's test was negative, however, after hydrolysis with 10 per cent sulfuric acid. The Schiff test was positive, but little significance can be attached to this,

since the virus contained lipids (positive acrolein test) as well as pentoses. The glyoxylic acid reaction was negative.

Dialysis against distilled water resulted in precipitation and loss of hemagglutinative activity of the virus. The salt-free materials frozen and dried *in vacuo* were insoluble in 0.9 per cent sodium chloride and Ringer's solution.

Quantitative Analysis—Concentrates obtained from large pools of starting material, as described above, and consisting of 100 to 200 mg. of the respective types of virus, were dialyzed against running distilled water at 2–5° for 72 to 96 hours. The materials were then frozen with dry ice and dehydrated *in vacuo* from the frozen state. The analytical methods used will be given in detail in the description of the analysis of the influenza virus A. The influenza virus B and the swine influenza virus were treated in an identical manner. The results of elementary and constitutional analyses of the virus and fractions are given in Table I.

Analyses for carbon, nitrogen, and phosphorus were made on the whole virus complex. For this, samples of 5 to 7 mg. of the frozen and dried whole virus complex were weighed into small Pyrex boats. After the material was dried to constant weight at 40° over P_2O_5 , the weights were taken in closed weighing bottles. Carbon was determined by wet combustion (6), nitrogen by the semimicro-Kjeldahl method with the digestion mixture described by Kirk, Page, and Van Slyke (7) and 0.01 N HCl for collection of the distillates and 0.01 N NaOH for titration, and phosphorus by the method of Kirk (8).

Fractionation with Lipid Solvents—Samples of about 50 mg. of whole virus were dried to constant weight as described above. The dried sample was transferred to a 40 ml. extraction tube which was fitted by means of a ground glass connection to a Graham condenser and allowed to reflux for 2 hours with 20 ml. of a mixture of absolute alcohol (3 parts) and ethyl ether (1 part). All lipid solvents were freshly distilled within a few days of use. The extract and all of the alcohol-ether-insoluble material were transferred quantitatively to a tared sintered glass crucible equipped with a suction lead and a ground glass joint fitting into a 50 ml. volumetric flask. The extraction tube was washed with successive portions of alcohol ether, which were then drawn through the precipitate in the sintered glass crucible into the flask. The alcohol-ether-insoluble fraction was dried to constant weight on the crucible for analysis, as described in a subsequent section.

A 5 ml. aliquot of the 50 ml. alcohol-ether-soluble fraction was transferred to a tared weighing bottle, evaporated, and the dry weight determined. From this value the percentage by weight of the alcohol-ether-soluble material was found. The carbon of the alcohol-ether-soluble material was determined in 10 ml. aliquots and nitrogen was determined in 50 ml. aliquots, according to the method of Kirk, Page, and Van Slyke (7).

TABLE I

Chemical Analysis of Influenza Viruses A (PR8 Strain) and B (Lee Strain) and Swine Influenza Virus

		Influenza virus A		Influenza virus B		Swine influenza virus	
		Per cent of whole complex	Per cent of fraction	Per cent of whole complex	Per cent of fraction	Per cent of whole complex	Per cent of fraction
Whole virus complex	Carbon	53.2		52.7		51.4	
	Nitrogen	10.0		9.7		9.0	
	Phosphorus	0.97		0.94		0.87	
	Carbohydrate (as glucose)	12.5		13.1		10.0	
	Desoxyribonucleic acid	2.1		3.7		+	
Alcohol-ether-soluble fraction	By weight	32.4		30.0		30.1	
	Carbon	19.1	59.0	18.3	61.0		
	Nitrogen	0.86	2.65	0.73	2.43		
	Carbohydrate* (as glucose)	7.1	21.8	7.7	25.8		
	Non-lipid material	8.79	26.5	7.0	23.0	6.1	20.0
Petroleum ether-soluble fraction	Total lipid by weight	23.8		23.0			
	Total lipid by analysis	23.4		22.4		24.0	
	Carbon	17.2	72.3	16.0	69.6	17.7	73.8
	Nitrogen	0.74	3.1	0.49	2.13	0.43	1.86
	Phosphorus	0.48	2.0	0.48	2.10	0.45	1.95
Non-lipid fraction	Phospholipid	11.3	48.3	11.23	50.6	10.67	44.4
	Cholesterol (total)	7.0	30.1	3.68	16.4	5.7	23.6
	Neutral fat	5.1	21.6	7.17	32.0	7.7	32.0
	Alcohol-ether-insoluble	68.5		69.4		71.6	
	Total non-lipid†	77.5		76.4		77.6	
	Carbon	33.3	48.6	33.2	47.8		
	Nitrogen	9.17	13.4	9.10	13.1	8.78	11.3
	Phosphorus	0.47	0.68	0.42	0.60	0.48	0.62
	Carbohydrate (as glucose)	7.3	10.7	9.42	13.56		
	Desoxyribonucleic acid	1.51	2.20	1.21	1.75	+	

All values are in per cent of the dry weight of the whole complex or fraction

* Very labile, see the text

† Sum of the alcohol-ether-insoluble material and the alcohol-ether-soluble non-lipid material

To separate the true lipid from the non-lipid alcohol-ether-soluble material, a 25 ml aliquot of the alcohol-ether extract was transferred to a beaker, evaporated at 58°, and extracted with redistilled petroleum ether

which had been washed with concentrated sulfuric acid. The petroleum ether extract was then filtered into a 25 ml volumetric flask. A 5 ml aliquot of the extract was transferred to a tared weighing bottle, and the dry weight of the true lipid (petroleum ether-soluble) fraction determined. From this value the total true lipid by weight was found, and by subtraction the amount of non-lipid material in the alcohol-ether-soluble fraction was calculated.

1 ml aliquots of the petroleum ether-soluble fraction were evaporated to dryness over the water bath, and the total true lipid carbon was determined by combustion manometrically according to Kirk, Page, and Van Slyke (7) and Van Slyke and Folch (6). Lipid nitrogen was determined by the method of Kirk, Page, and Van Slyke (7). Analysis for phosphorus was made by the method of Kirk (8).

Total cholesterol was determined (7) on the alcohol-ether extract. For this 40 ml aliquots of the alcohol-ether-soluble fraction were saponified for 2.5 hours at 85° by the addition of 10 ml of saturated alcoholic solution of NaOH. The saponified materials were extracted with petroleum ether, and the extracts were collected in a 25 ml volumetric flask. Cholesterol was precipitated as the digitonide from 10 ml aliquots of the extracts, collected and washed on filter sticks, and the carbon dioxide of the digitonide-cholesterol complex was determined manometrically.

The phospholipid content was calculated from the value obtained for lipid phosphorus determined in the petroleum ether extract.

The total lipid by analysis and the neutral fat were estimated from the values of total lipid carbon, phospholipid, and cholesterol, as described by Kirk, Page, and Van Slyke (7).

Carbohydrate—The results of the Molisch tests showed the presence of bound carbohydrate released only after hydrolysis. The presence of pentose was evident from the positive test with orcinol and sulfuric acid and with Bial's reagent. After hydrolysis with 10 per cent sulfuric acid, the Bial's test became negative, indicating that the carbohydrate (pentose) detected was desoxypentose. No evidence was seen of ribose.

Total carbohydrate of the whole virus complex was determined by means of the orcinol and sulfuric acid method (9), with glucose as standard. In the case of influenza virus B (Lee strain) carbohydrate was determined also by the carbazole method (10). Total carbohydrate was determined also on the alcohol-ether-soluble fraction, and on the alcohol-ether insoluble material (non-lipid fraction).

Nucleic Acid—The occurrence of desoxypentose suggested the presence of nucleic acid of the type containing this sugar. Analyses were made for the content of desoxyribonucleic acid in the whole virus complex and in the non-lipid fraction. For this the diphenylamine reaction of Dische (11)

was used with the precautions described by Seibert (12) and by Snell and Snell (13). The standard for comparison was a preparation of thymonucleic acid containing 9.4 per cent phosphorus.

Extraction of Active Purified Virus Concentrate with Petroleum Ether—An experiment was done in order to establish whether or not the lipid portion of the virus was an integral part of the whole complex. A concentrate containing 40 mg (Kjeldahl N \times 100) of influenza virus A in Ringer-calcium chloride solution was divided into two portions, 6 ml for extraction and 4 ml for the control. One of these was extracted five times successively at 25° with 6 ml portions of freshly redistilled petroleum ether, previously washed with concentrated sulfuric acid. The virus solution and each portion of petroleum ether were shaken together in a test-tube with a whirling motion. For the first extraction, shaking was continued for 15 minutes and for the others 5 minutes each. The emulsion formed during the extractions was broken by light horizontal centrifugation. The petroleum ether layer was carefully syphoned into a 25 ml volumetric flask with gentle suction. Most of the excess petroleum ether was drawn off from the extracted sample by evacuating it several times to the point at which the virus solution began to bubble. There was little, if any, difference in the appearance of the extracted and unextracted samples. Samples of the extracted and the control materials were titrated for infectivity for chick embryos and for hemagglutinative activity, and were examined by the scale method in the analytical ultracentrifuge. The petroleum ether extract was made up to 25 ml (some of the petroleum ether was lost by evaporation in the manipulation), and the lipid carbon in 10 ml aliquots was determined manometrically.

The amount of lipid extracted in this manner represented 0.34 per cent of the whole virus complex. If the total lipid content of the virus is taken as 2.4 per cent (Table I), the amount of lipid extracted directly with petroleum ether constituted 1.4 per cent of the total lipid. The hemagglutinative capacity of the petroleum ether-extracted virus was 30 per cent less than that of the control material, while the infectivity of the extracted virus declined approximately one 5-fold dilution. Definite, though not pronounced, change in the physical character of the virus was seen in the sedimentation diagram, which showed a greater spread in the sedimenting boundary after extraction with petroleum ether. There was no change in sedimentation constant.

DISCUSSION

The results of the analyses of the three types of influenza virus are given in Table I. On all of the samples chosen for study, examinations were made with the analytical ultracentrifuge, the Lamm scale method &

used, and with the electron microscope. The results indicated a high degree of homogeneity of the preparations with respect to particle kind. These data obtained on ultracentrifugation and with the electron microscope will be described in detail in a subsequent publication.¹ It was apparent, therefore, that the findings of chemical analysis represented the properties of the influenza viruses. It should be emphasized that the materials studied were obtained in relatively small quantities by carefully controlled purification procedures, and that no specimen inhomogeneous by the methods described was included.

From the results tabulated, the three types of influenza virus appeared closely similar in general constitution. In each case the purified components were readily separable into two fractions, a lipid constituent soluble in petroleum ether and another which was insoluble in petroleum ether. The proportions of these fractions were similar for the three types.

In view of the proportion of lipid and non-lipid material found in preliminary analysis of the materials, the contents of carbon and nitrogen in the whole virus complex at first appeared low. With the subsequent finding of the unexpectedly high content of carbohydrate in the whole virus complex, both carbon and nitrogen values were observed to be compatible with the determined proportions of lipid, non-lipid, and carbohydrate constituents. With the finding of this quantity of carbohydrate, however, the non-lipid phosphorus content of the whole virus complex became at once incompatible with the concept that the carbohydrate was bound in nucleic acid, which has been the case for other animal and plant viruses examined. In this relation, if all of the non-lipid phosphorus of the whole virus were contained in nucleic acid, the content of carbohydrate with it would not be more than about 3 per cent. This quantity would represent only 25 per cent of the carbohydrate found in the whole virus complex.

Approximately like proportions of alcohol-ether-soluble and insoluble materials were found under comparable conditions in each type of virus. In addition to lipid, the alcohol-ether-soluble fraction contained a relatively large amount of carbohydrate, a constitution compatible with the carbon value obtained for this fraction. That most of the petroleum ether insoluble material of the alcohol-ether-soluble fraction was carbohydrate was indicated by the fact that the percentages of the fractions calculated from the carbohydrate determinations were close to those obtained by the differences in the weights of the respective alcohol-ether and petroleum ether-soluble fractions. The alcohol-ether-soluble nitrogen values were higher than those obtained for the petroleum ether-soluble fractions.

¹ Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., and Beard, J. W., in preparation.

This could be attributed either to the presence of amino acids in the alcohol-ether-soluble fraction or to the possibility that nitrogen was present in the carbohydrate. It was also possible that during the alcohol-ether extraction nucleic acid was broken down, since the nucleic acid values obtained for the alcohol-ether-insoluble fraction were lower than those obtained for the whole complex.

The possibility of loss of nucleic acid in extraction with alcohol-ether was supported more directly in other experiments. When 50 ml aliquots of the alcohol-ether-soluble fractions of influenza viruses A and B were evaporated to dryness and carbohydrate determination attempted with sulfuric acid and orcinol, the acid solutions became yellow in color as soon as the cold acid and samples were mixed. Upon addition of orcinol and heating in the water bath at 100° for 10 minutes, the color developed very rapidly in contrast to the usual slow development of color with samples of the whole virus and of the alcohol-ether-insoluble fraction. It will be noted that the sum of the carbohydrate values for the alcohol-ether-soluble fraction and the alcohol-ether-insoluble fraction was greater than the values obtained for the whole virus complex. It was obvious that the carbohydrate in the alcohol-ether-soluble fraction was much more labile than that in either of the other two fractions, or that other substances giving color with orcinol were interfering with the reaction. Desoxyribose is an extremely labile carbohydrate. Gurn and Hood (10) found that, when samples of desoxyribose were mixed with sulfuric acid, an intense yellow color was produced. They found also that contamination of ribonucleic acid with small quantities of desoxyribonucleic acid gave rise to high carbohydrate values for the former.

The petroleum ether-soluble or true lipid fractions of the influenza virus A and the swine virus constituted approximately the same percentages of these two viruses. The influenza virus B tended in general to give lower values for lipid, as indicated.

That practically all of the lipid was an integral part of the virus complex was indicated by the results of extraction of the whole wet virus with petroleum ether, since only a very small proportion of lipid material was removed by the procedure. At the same time some inactivation was observed, and it was thus possible that the small quantity of fat obtained came from degraded virus particles and was itself a part of the virus complex. If the lipid were associated with the virus as an impurity which was carried through the purification process or was in loose union (adsorbed on the particles), extraction with petroleum ether would be expected to remove it. It would appear that the lipid associated with the virus, in all cases in definite proportions in petroleum ether extracts of the alcohol-ether-soluble material, was actually bound in chemical union as a part of the complex,

since it was only by drastic treatment with alcohol-ether that it was removable

Examination of the petroleum ether-extracted concentrate in the analytical ultracentrifuge revealed a slight increase in particle heterogeneity, though there was no change in sedimentation constant. It should be noted that any marked change in the lipid content of the virus in this preparation would be expected to result in considerable change in the sedimentation constant. More pronounced evidence of change in the virus due to exposure to the petroleum ether was seen in the decline of biological activity.

The cholesterol content of the influenza virus B was definitely lower than that of the other two types. This low cholesterol content was further substantiated by analysis of another alcohol-ether fraction of influenza virus B, which gave a value of 4.2 per cent cholesterol. The phospholipid of the swine virus appeared to be significantly lower than that of the other two types, and this is reflected in the phosphorus values of the respective total virus complexes. The variations in neutral fat were natural reflections of the variations in phospholipid and cholesterol.

The atomic ratios of nitrogen to phosphorus in the lipid fractions were of interest. The influenza virus B and the swine virus contained 2 atoms of lipid nitrogen to 1 of phosphorus, indicating the presence of a diamino monophosphatide. This was similar to the findings with the Eastern (14) and Western² strains of equine encephalomyelitis virus and was in direct contrast to the findings with the normal chick embryo component (15), and the elementary bodies of vaccinia (16), which contain lipid nitrogen and phosphorus in a 1:1 ratio. The lipid nitrogen of the influenza virus A was higher than that of either of the other two types. Whether or not this represented a different type of phospholipid was not further investigated. The nitrogen value for the petroleum ether-soluble fraction of the swine virus was somewhat lower than that found for the other two types, a finding consistent with the elementary analysis of the whole swine virus and the content of phospholipid.

The alcohol-ether-insoluble fractions contained 68.5, 69.4, and 71.6 per cent of the three types of influenza virus. To these values were added the differences in the weights of the respective alcohol-ether-soluble and petroleum ether-soluble fractions, giving values which were expressed as total non-lipid material. These percentages, 77.5 for influenza virus A, 76.4 for influenza virus B, and 77.6 for the swine virus, represent the combined protein-carbohydrate material. If the percentages of total virus carbohydrate are subtracted from these values, an estimate of the probable protein content of the respective types is obtained. These values

- Taylor, A. R., Sharp, D. G., Beard, D., and Beard, J. W., unpublished data

would be 65.0 per cent for influenza virus A, 63.6 per cent for influenza virus B, and 67.6 per cent for the swine influenza virus.

In the present work no evidence has been seen of the presence of ribose. Qualitative tests have been invariably positive for desoxyribose and desoxyribose- or thymonucleic acid. As stated in previous communications, the non-lipid phosphorus content would represent 4.3 to 5.0 per cent of nucleic acid if all of this phosphorus were present in the form of nucleic acid. The content of desoxyribonucleic acid in the whole virus, as determined by the diphenylamine reaction, fell considerably below these values. In addition, the percentages of desoxyribonucleic acid found in the alcohol-ether-insoluble fraction are markedly below those obtained on the whole virus complex. In the case of the swine virus, quantitative determination was not made, but the test for thymonucleic acid was positive. It was pointed out above, under the discussion of the alcohol-ether-soluble fraction, that carbohydrate in a labile form was apparently freed from the virus complex during the extraction with alcohol-ether. It was also noted that desoxyribose in the free form reacts with carbazole and orcinol in such a manner that it gives an intense color and high values. The carbohydrate values obtained with orcinol for the alcohol-ether-insoluble fraction, namely 7.3 per cent for influenza virus A and 9.4 per cent for influenza virus B, were much higher than can be accounted for as carbohydrate present in the form of nucleic acid. It was evident that not only was much of the carbohydrate of the viruses present in complex form in a union other than with nucleic acid, but also that there was a strong possibility that a considerable proportion of the non-lipid phosphorus, as well, was not in the form of nucleic acid phosphorus. With Schiff's reagent, which, in the absence of lipids and extraneous aldehydes, is considered specific for thymonucleic acid, the alcohol-ether-insoluble fraction of the influenza virus A gave a weakly positive test.

The nature of the carbohydrate in the influenza virus other than that bound as desoxyribose in nucleic acid has not been determined. When extinction coefficient ratios ($\epsilon_{520}/\epsilon_{540}$) were obtained by the carbazole method according to Gurin and Hood (10), values of 1.28 and 1.35 resulted for influenza virus B. This suggested the possible presence of mannose or glucose-galactose in complex form, or both. The mucic acid test was positive, giving a further indication of galactose.

SUMMARY

Elementary and constitutional analyses were made on purified influenza virus A (PR8 strain), influenza virus B (Lee strain), and the swine influenza virus. The respective particulate materials were found to be essentially similar complexes of protein, lipid, and carbohydrate, containing small

amounts of nucleic acid of the desoxyribose type. Small differences which appeared significant were observed in the total lipid and in the proportions of the lipid constituents. The total lipid content of the influenza virus A and the swine influenza virus was approximately the same, 24 per cent of the whole complex. The influenza virus B consistently yielded lower total lipid values. The phospholipid content of the swine virus was definitely lower than that of the influenza virus A and influenza virus B. The total cholesterol content of the influenza virus B was 30 to 40 per cent lower than the values obtained for the other two types. The lipid obtained by fractionation of the dehydrated virus appeared to be an integral part of the virus complex, since only a very small amount of fat was obtained by extraction of fully active virus with petroleum ether. Of especial interest was the content of carbohydrate, which was much larger than could be accounted for in combination in nucleic acid.

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LETTERS TO THE EDITORS

IDENTIFICATION OF FOLIC ACID AS ONE OF THE UNKNOWN DIETARY ESSENTIALS FOR GUINEA PIGS

Sirs

The recognition of three new dietary essentials required by guinea pigs in addition to the known vitamins was reported from this laboratory in 1942¹ and subsequently by others². Two of these factors were shown to be present in linseed oil meal. We now wish to report the successful substitution of folic acid for one of the linseed factors.

In attempts to isolate the factor GPF-2, it was found that this could be replaced by a mixture of cellulose and increased casein in the diet. Since concentrates of GPF-2 contained both fiber and protein, it was felt that the activity of the concentrates was due to the presence of these materials. About this time Hogan and Hamilton³ used cellulose, and Sober *et al*² recommended a high protein level in the diet of guinea pigs.

With GPF-2 supplied as known materials, further attempts were made to isolate GPF-1. The basal ration contained glucose 45 gm, vitamin-free casein (Labco) 30 gm, cellulose 20 gm, salts⁴ 5 gm, and the vitamins as used previously¹. Good growth was obtained on this ration plus solubilized liver extract (Wilson's Fraction L⁵). Although this fraction was a rich source of folic acid, concentrates of folic acid prepared from it⁶ were not effective *per se*. Furthermore a folic acid-free fraction prepared by treating Fraction L with lead acetate and norit likewise was ineffective by itself. However, the lead acetate-norit preparation plus the folic acid concentrate permitted good growth and survival. Finally, through the courtesy of

¹ Woolley, D. W., *J. Biol. Chem.*, **143**, 679 (1942).

² Sober, H. A., Mannering, G. J., Cannon, M. D., Elvehjem, C. A., and Hart, E. B., *J. Nutr.*, **24**, 503 (1942).

³ Hogan, A. G., and Hamilton, J. W., *J. Nutr.*, **23**, 533 (1942).

⁴ Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).

⁵ We wish to thank Dr. David Klein for gifts of this material.

⁶ Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, **141**, 521 (1941).

Dr E L R Stokstad⁷ and Dr A D Emmett,⁸ crystalline folic acid (vitamin B₁₂) was tested in place of the folic acid concentrate. The crystalline vitamin proved effective (see the table).

We regard folic acid to be identical with GPF-1, since our previous preparations of this factor¹ were rich in folic acid (*Lactobacillus casei* test) and

Effect of Folic Acid (FA) and Lead Acetate-Norit Preparation (LAN) from Solubilized Liver Extract on Growth and Survival of Guinea Pigs

Addition to basal	Amount added	Folic acid	No of animals	Survivors after 4 wks	Average gain
	<i>per cent of ration</i>	<i>γ per day</i>			<i>gm per wk</i>
None		0	11	1	
Solubilized liver	8	10	17	17	25
FA concentrate		13	5	0	
LAN	Equivalent to 8	0.5	12	0	
FA concentrate + LAN	" " 8	13.5	11	11	28
Crystalline FA + LAN	" " 8	6.5	4	4	23

because concentrates of folic acid and finally the pure substance replaced the factor. The lead acetate-norit preparation may be regarded as a concentrate of the new dietary essential, GPF-3, for which Wilson's Fraction L appears to be a good source.⁹

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⁷ Stokstad, E L R, *J Biol Chem*, **149**, 573 (1943)

⁸ Pfaffner, J J, Binkley, S B, Bloom, E S, Brown, R A, Bird, O D, Emmett, A D, Hogan, A G, and O'Dell, B L, *Science*, **97**, 404 (1943)

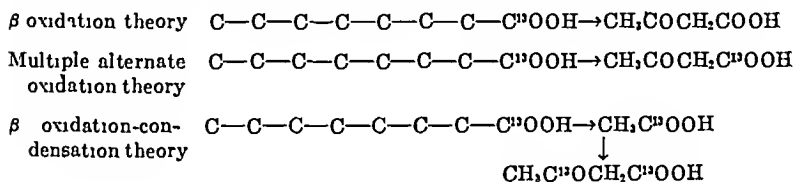
⁹ Mannering, G J, Cannon, M D, Barki, H V, Elvehjem, C A, and Hart, E B, *J Biol Chem*, **151**, 101 (1943)

¹⁰ Nutrition Foundation Fellow

THE MECHANISM OF FATTY ACID OXIDATION

Sirs

Despite considerable study the mechanism of the oxidation of fatty acids is still obscure. It occurred to us that information concerning this problem could be obtained by studying the breakdown of a typical fatty acid (e.g. *n*-octanoic) labeled by the incorporation of C^{13} in the carboxyl group. The distribution of C^{13} in the intermediate ketone bodies as predicted for the prevalent theories concerning fatty acid breakdown are as follows



According to the β oxidation theory the ketone bodies are formed from the terminal 4 carbons of the fatty acid chain and hence would contain no excess isotope. According to the multiple alternate oxidation hypothesis,¹ in which it is presumed oxidation occurs at alternate carbon atoms prior to splitting into 4-carbon units, the resulting acetoacetic acid should contain excess C^{13} only in the carboxyl group. If the ketone bodies arise by a random condensation of 2-carbon units formed through β oxidation,² the ketone bodies should contain the excess isotope distributed evenly between the carbonyl and carboxyl carbons.

n-Octanoic acid containing 5.5 per cent of C^{13} in the carboxyl group was incubated with rat liver slices and the acetoacetic acid thus formed broken down further by heating, according to the equation, $CH_3COCH_2COOH = CH_3COCH_3 + CO_2$.

The isotope content of each fraction was determined by analysis in the mass spectrometer (a commercial instrument manufactured by the Consolidated Engineering Corporation). The values obtained, compared with those predicted by the three theories mentioned above, are shown in the accompanying table. In adjoining columns are given the actual percentages and the excess over the normal complement of C^{13} , which for our instrument was found to be 1.04 atoms per cent.

The results clearly indicate that at least in the liver the oxidation of fatty

¹ Jowett, M., and Quastel, J. H., *Biochem. J.*, **29**, 2159 (1935).

² MacKay, E. M., Wick, A. N., Carne, H. O., and Barnes, C. P., *J. Biol. Chem.*, **138**, 63 (1941).

acid proceeds by a mechanism involving splitting into 2-carbon fragments, followed by condensation to the ketone bodies. This investigation is being continued with other tissues and other fatty acids.

We wish to acknowledge with thanks the generosity of H. C. Urey in supplying us with a substantial amount of one of his carbon isotope con-

Distribution of C¹³ in Acetoacetic Acid Formed by Breakdown of Carboxyl-Labeled n-Octanoic Acid

	C ¹³ content, atoms per cent*			
	Acetone		Carboxyl	
		Excess		Excess
Observed	1.34	0.30	1.89	0.85
β oxidation-condensation theory	1.41	0.37	2.14	1.10
Multiple alternate oxidation theory	1.04	0	3.24	2.20
β oxidation theory	1.04	0	1.04	0

* The C¹³ content is expressed as atoms per cent calculated from the equation

$$\text{Atoms \% C}^{13} = \frac{\text{moles C}^{13}}{\text{moles C}^{12} + \text{moles C}^{13}} \times 100$$

centrates without which this work would have been impossible. We also express our thanks to the Houdry Laboratories of the Catalytic Development Corporation of Marcus Hook, Pennsylvania, for its active collaboration.

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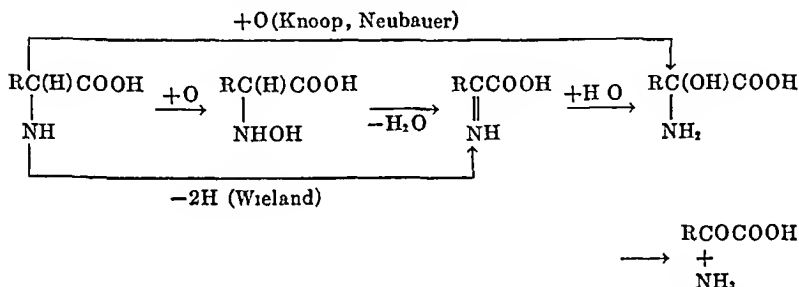
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GRACE MEDES
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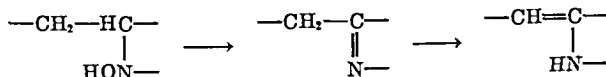
N-HYDROXY- α -AMINO ACIDS AS POSSIBLE INTERMEDIATES IN THE OXIDATIVE DEGRADATION OF α -AMINO ACIDS

Sirs

Both Knoop¹ and Neubauer² assumed that the oxidative degradation of α -amino acids to α -keto acids involved only one step, namely the direct formation of α -hydroxy- α -amino acids, which on loss of ammonia yield the α keto acids. Later, Wieland and Bergel³ advanced the theory that the α -amino acids are dehydrogenated to α -imino acids, which on hydration are converted into the α -hydroxy- α -amino acids. Another possibility is that α -amino acids are oxidized to N-hydroxy- α -amino acids, the latter then losing the elements of water to give α -imino acids.



N-Hydroxy-*dl*- β -phenylalanine was prepared according to Traube⁴. M p 164-165°, decomposition (corrected), Traube found 157-158°. Analysis, calculated, C 59.66, H 6.12, found, C 59.74, H 6.16. When heated with 2 equivalents of aqueous 0.1 N hydrochloric acid, it decomposed to phenylacetaldehyde, carbon dioxide, and ammonium chloride. However, when treated with acetic anhydride and pyridine, it lost its N-hydroxyl group together with the α -hydrogen atom and gave α -acetimino- β -phenylpropionic acid in the form of the tautomeric α -acetaminocinnamic acid in accordance with the scheme,



¹ Knoop, F, *Z physiol Chem*, **67**, 498 (1910) Knoop, F, and Kertess, E, *Z physiol Chem*, **71**, 261 (1911)

² Neubauer, O, and Fromherz, K, *Z physiol Chem*, **70**, 338 (1910-11)

³ Wieland, H, and Bergel, F, *Ann Chem*, **439**, 196 (1924)

⁴ Traube, W, *Ber chem Ges*, **28**, 1794, 2301 (1895)

Rapid N-acetylation followed by azlactone formation prevented decarboxylation of the N-hydroxy- α -amino acid or of the α -imino acid

Acetic anhydride (20 gm) was added to a suspension of N-hydroxy *dl*- β -phenylalanine (3.62 gm) in pyridine (20 gm). There was an immediate reaction. The solution was heated to 90° for 4½ hours and evaporated to dryness under reduced pressure. The azlactone was dissolved in boiling 67 per cent acetic acid to open the ring. The reagent was distilled off under reduced pressure and the residue recrystallized from ethyl acetate. Yield, 65 per cent (2.67 gm) of α -acetaminocinnamic acid, m.p. and mixed m.p. 193° (corrected). This on boiling with 1 N hydrochloric acid yielded phenylpyruvic acid, in known fashion.

Thus the convertibility of an N-hydroxy- α -amino acid into an α keto acid through the α -imino acid has been demonstrated *in vitro*. If N-hydroxy- α -amino acids are the actual precursors in the oxidative deamination of α -amino acids, the oxidative deaminase would be dehydrative as well.

The work on N-hydroxy- α -amino acids is being continued.

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